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Studies on Corynebacterium Enzymicum (Mellon) Eberson.

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STUDIES ON CORYNEBACTERIUM ENZYMICUM (MELLON) EBERSON.

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STUDIES ON *CORYNEBACTERIUM ENZYMICUM* (MELLON) EBERSON

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ABSTRACT

The original isolate described as Bacillus enzymicus by Mellon and designated Corynebacterium enzymicum by Eberson is no longer available. The two strains isolated by Dr. L. H. Schwarz (ATCC 8155 and 8156) and identified by him as C. enzymicum were employed in this attempt to more accurately classify this species.

The Schwarz strains agree quite well with the description of Mellon's organism except that the dimorphism (rods to cocci to rods) reported by Mellon was not observed, and the pathogenicity for rabbits was not confirmed. The optimum growth temperature was found to be 30-33 C rather than 37 C as reported by Mellon.

C. enzymicum differs from the type species, C. diphtheriae, in several ways. Morphologically, C. enzymicum is a small coccoid bacillus, quite uniform in size and lacking the irregularity in shape characteristic of the type species. C. enzymicum is highly fermentative and totally indifferent to oxygen in contrast to C. diphtheriae which is essentially oxidative. The former has neither oxidase, cytochrome oxidase or catalase, while the latter possesses all these enzymes. C. enzymicum produces large amounts of lactic acid from glucose, C. diphtheriae produces lactic acid only in traces or none. The diphtheria organism grows readily without sugar, while C. enzymicum requires a fermentable carbohydrate for good growth.

Morphologically, C. enzymicum bears some resemblance to members of the genus Microbacterium, but it differs from that genus in its fermentative character, high yield of lactic acid from glucose, dependence

on carbohydrate for growth and lack of heat resistance. Microbacterium species possess catalase and cytochrome oxidase; both are lacking in C. enzymicum.

C. enzymicum has much in common with the homofermentative members of the genus Lactobacillus. Both produce almost the theoretical yield of lactic acid from glucose, with only traces of other substances; both require carbohydrate for growth, are strictly fermentative and do not possess catalase and cytochrome oxidase. The acid produced by C. enzymicum is dextrorotatory [L(+)-lactic acid], and the optimum growth temperature of 30-33 C places this organism very close to Lactobacillus casei. The G-C content (mole % G + C) of the DNA of C. enzymicum was found by the method of Marmur (modified) and Bendich to be 39. This value approximates that of Lactobacillus acidophilus and several other lactic acid bacteria, and is considerably lower than that for any of the corynebacteria.

It is recommended that this species be removed from the genus Corynebacterium and placed in the genus Lactobacillus as Lactobacillus enzymicus. It is further recommended that the Schwarz strain C-1-a, (ATCC 8156) be designated as the neotype for this species.

INTRODUCTION

The genus Corynebacterium, originally created by Lehmann and Neumann (1896), now consists of a large group of microorganisms of both animal and plant origin having diverse characteristics. Clark (1952), stated that the genus "Corynebacterium as currently defined has become an undesirable and unwieldy genus". There are now twenty-eight aerobic species and five anaerobic species of the genus recognized in Bergey's Manual (Breed et al., 1957). Both motile and non-motile, gram-positive and gram-negative (Breed et al., 1957; Carrier and McCleskey, 1961), and even denitrifying (Hart, Larson and McCleskey, 1965) and catalase-negative (Carrier, 1963) organisms are encountered in this genus. Consequently a problem is posed to the taxonomists to reclassify these organisms, which have little resemblance to the type species.

Carrier (1963) made an extensive survey of forty-four cultures, representing twenty-three of the twenty-eight aerobic species of the genus Corynebacterium. He found that C. enzymicum was quite unlike the type species, in that it was the only species which was catalase-negative and lacked the cytochrome oxidase system. The other corynebacteria were highly oxidative, and produced little acid; C. enzymicum was strongly fermentative, and produced considerable acid from carbohydrates. It was very indifferent to oxygen and it acidified and coagulated milk. Carrier suggested that C. enzymicum should be removed from the genus Corynebacterium and placed in the genus Lactobacillus.

In view of the above facts, it was considered desirable to examine authentic cultures of this organism. However, the original strain of C. enzymicum isolated by Mellon (1916) is no longer available. The culture designated as C. enzymicum, strain 8155, studied by Carrier (1963), was isolated by Schwarz in 1940. The strain 8155 is a variant of the parent strain 8156 which was also isolated by Schwarz. Both of these strains are maintained in the American Type Culture Collection. These two strains were utilized in a study of their physiological and biochemical characteristics, and they were compared with the type species of the genera Corynebacterium and Microbacterium, and with a representative member of the genus Lactobacillus.

REVIEW OF LITERATURE

Corynebacterium enzymicum

Isolation. Mellon (1916) first isolated *C. enzymicum* from the lungs of a patient who complained of a chronic cough and loss of weight. He isolated the organism on blood agar, and observed a very fine dew-drop growth in six days. The organism stained well with Loeffler's methylene blue and was strongly gram-positive. It was definitely pleomorphic and showed coccoid forms. Mellon (1917) considered this organism to be an unusual diphtheroid bacillus and named it *Bacillus enzymicus*.

Eberson (1918) studied Mellon's organism and renamed it *Corynebacterium enzymicum*.

Schwarz (1941) isolated an organism which he considered to be *C. enzymicum* from a cockroach (*Blatella germanica*) obtained from the cellar of a man who was in the habit of brewing beer for his own consumption. He designated this organism *C. enzymicum*, strain C-1-a, and isolated a variant (strain C-1) from his original strain. However, no published report on Schwarz's work is available in the literature.

Original controversy. Mellon (1917) reported that *B. enzymicus* (*C. enzymicum*) was a diphtheroid bacillus when grown on solid media, and a coccus when grown in liquid media. Mellon described a method whereby he could transform the bacillary type into a coccus and back again. He noticed that the change in form of the organism from a barred bacillus to the coccoid or diplococcus form was accompanied by

a cultural change from a slightly flocculent growth to a very luxuriant, diffuse, finely granular growth.

Subsequently, much controversy arose over Mellon's report. Eberson (1918) studied Mellon's B. enzymicus and concluded that diphtheroids conform to the fundamental laws of bacteriology and are not readily transformed into cocci and back again. He isolated two distinct biotypes from a culture of Mellon's B. enzymicus. The coccus form associated with a bacillary diphtheroid was antigenically distinct from the bacillus. Eberson concluded that Mellon's culture was a mixture of two bacteria, a bacillus and a coccus. His photographs also seemed to indicate that Mellon's culture was not pure.

Mellon (1920) studied a pure culture started from a single cell and confirmed his earlier work. He attributed the various morphological changes in B. enzymicus to "response of a sensitive organism to a medium of varying chemical constitution".

Morphological characteristics. According to Mellon (1917), C. enzymicum forms beaded and club-shaped rods. They are definitely pleomorphic and show coccoid forms. Carrier (1963) reported the C. enzymicum studied by him to be a very short coccoid rod having a tendency to form aggregates of cells on all media. Mellon (1917) reported that C. enzymicum was strongly gram-positive and stained well with Loeffler's methylene blue showing a barred appearance. Carrier (1963), on the other hand, found that C. enzymicum (Schwarz strain, C-1) did not stain readily with methylene blue, crystal violet, malachite green or carbol fuchsin. Mellon (1917) reported that the bacillary form retained the carbol fuchsin when decolorized

in weak acid-alcohol (1% HCL in 20% ethanol), and that the coccoid form did not hold the stain. Carrier (1963) found that the organism was not acid-fast by the Ziehl-Neelsen procedure. According to Mellon (1917), C. enzymicum is non-motile and non-spore forming. Capsules were not seen in laboratory cultures but were demonstrated in vivo. Carrier (1963) reported that fat globules could be seen when the organisms were grown on a medium containing glycerol and stained with Sudan black B.

Cultural characteristics. The bacillary form develops very small, transparent colonies on blood agar in twenty-four to forty-eight hours. Finally the colonies become translucent to grey; the coccoid form develops luxuriant, coalescing, yellowish white, moist growth on any medium (Mellon, 1917). Carrier (1963) reported the development of very small (approximately 1.0 to 0.5 mm in diameter) colonies on trypticase soy agar containing 1% bovine serum. Mellon (1917) found that the bacillary form produced sparse, granular growth and sediment and the coccoid form produced diffuse, luxuriant growth in glucose broth. Carrier (1963) reported the production of even turbidity with sediment in liquid media.

Mellon (1917) found that the bacillary form had fastidious cultural characteristics, while the diplococcus form was very adaptable. Gelatin was not liquefied but it supported the growth of C. enzymicum.. Potato did not support growth. Animal fluids with the exception of ascitic fluid facilitated its growth. All media gave better growth with the addition of 1% glucose (Mellon, 1917). Carrier (1963) reported that C. enzymicum (Schwarz strain, C-1) did not form any pigment on any medium tested.

The effect of physical and chemical factors. C. enzymicum is facultative (Mellon, 1917). Carrier (1963) reported the organism to be markedly indifferent to oxygen; it did not grow at 4 C and 45 C but grew slowly at room temperature and at 40 C with the optimum growth at 37 C. Mellon (1917) reported that C. enzymicum had the intense viability characteristic of the diphtheroid group. He found the organism extremely resistant to desiccation and to the addition of antibiotic substances in the medium. It was not killed by prolonged exposure to sunlight. Its thermal death time was 58 to 60 C for three minutes. Carrier (1963) found that the organism tolerated 5.5% sodium chloride but did not grow in the presence of 2.5% bile salts and 0.0075% KCN.

Utilization of carbohydrates. C. enzymicum ferments glucose, maltose, dextrin (Mellon, 1917; Carrier, 1963), lactose (Mellon, 1917), ribose, xylose, arabinose, galactose, fructose, mannose, sucrose, trehalose, cellobiose, starch, salicin and pectin (Carrier, 1963). According to Mellon (1917), the fermentation of sucrose, raffinose, inulin, salicin, mannitol and glycerol is variable. The organism does not ferment lactose, rhamnose, melibiose, melizitose, raffinose, inulin, inositol, sorbitol, dulcitol and glycerol and utilizes mannitol only oxidatively (Carrier, 1963). The fermentation of carbohydrates by C. enzymicum is not associated with gas production (Mellon, 1917; Carrier, 1963).

Utilization of carbon and nitrogen compounds. C. enzymicum acidifies and coagulates milk (Mellon, 1917). Carrier (1963) reported that it did not utilize citrate, malonate, paraffin, asparagine , asparagine-glucose, leucine, alanine, lysine, tryptophane, histidine, aspartic acid, ascorbic acid and casein hydrolystate.

Biochemical characteristics. C. enzymicum does not liquefy gelatin and blood serum (Mellon, 1917). Carrier (1963) reported that the organism did not hydrolyze lecithin, tributyrin, urea, tyrosine, sodium hippurate, starch, cellulose, phenolphthalein phosphate and desoxyribonucleic acid. It hydrolyzed esculin and casein. According to Mellon (1917), C. enzymicum reduces only a slight amount of nitrate to nitrite. Carrier (1963) reported that it reduced tellurite, nitro blue tetrazolium, iodonitro tetrazolium, tetranitro blue tetrazolium and litmus but not methylene blue. It did not produce indol, hydrogen sulfide and acetylmethyl carbinol. It was strongly M.R. positive. It liberated ammonia from peptone but did not deaminate phenylalanine. It was non-hymolytic and coagulase-negative. According to Mellon (1917), C. enzymicum produces a slight amount of indol when grown in peptone water.

Presence of cytochrome oxidase and catalase. Carrier (1963) reported that C. enzymicum did not possess oxidase, cytochrome oxidase and catalase.

Pathogenicity. According to Mellon (1917), C. enzymicum is pathogenic to guinea pigs, rabbits and mice.

Characteristics of the genus Corynebacterium

Carrier (1963) concluded that C. enzymicum had little resemblance to the type species since it is strongly fermentative and lacks oxidase, cytochrome oxidase and catalase, and hence should be removed from the genus Corynebacterium. Possible considerations for its inclusion in the genus Lactobacillus or Microbacterium necessitates a discussion of the essential characteristics of these three genera, i.e., Corynebacterium, Lactobacillus and Microbacterium.

Morphology. According to Gilbert and Stewart (1927), Jensen (1934), and Conn and Dimmick (1947), the "classical" appearance of the corynebacteria is irregular morphology varying from long club- or wedge-shaped, occasionally branched rods, to short, almost coccoid cells. Metachromatic granules are considered by Denny (1902), Morse (1912), Eberson (1918) and Christensen (1949) characteristic of the type species although they may be absent in it, and often present in other species, and even in other genera. A feature that is apparently most typical of the whole group is the mechanism of cell division. Nakanishi (1901) pointed out that the cell division in corynebacteria results in an incomplete separation and an angular arrangement of the daughter cells. Hill and Rickards (1902) called this phenomenon "snapping division". Graham-Smith (1901) concluded that this phenomenon is characteristic of all corynebacteria. Jensen (1952) also asserted that this feature is a constant one that runs through the whole group. Non-motility has been regarded as one of the cardinal features of the genus Corynebacterium (Jensen, 1952), although motile species occur among the plant pathogens (Breed et al., 1957). According to Carrier and McCleskey (1961), the only motile species among the "animal group" is C. vesiculare which should be removed from the genus because it is gram-negative.

Conditions for growth. Carrier (1963) reported that all the plant pathogens examined by him were aerobic. All the animal species were aerobic also except C. diphtheriae, C. pseudotuberculosis, C. pyogenes, C. renale, C. kutscheri, C. striatum and C. enzymicum which were facultative. C. striatum and C. enzymicum were the most indifferent to oxygen.

Utilization of carbohydrates. The genus as a whole is highly oxidative. The fermentation of carbohydrates is apparently limited (Barratt, 1924; Frobisher, 1938; Welsch and Thibault, 1948). Glucose is metabolized by most of the species described as the animal group in Bergey's Manual (Breed et al., 1957), with the exception of C. equi, C. bovis and C. pseudodiphtheriticum (Brooks and Hucker, 1944; Merchant, 1935). The type species, C. diphtheriae, utilizes glucose, both fermentatively and oxidatively (Tasman and Brandwijk, 1938, 1940). Carrier (1963), however, reported that none of the carbohydrates was utilized fermentatively by the strains of C. diphtheriae which he tested. These strains utilized glucose and also ribose, xylose, fructose, mannose, maltose, trehalose, dextrin and glycerol only oxidatively. They did not utilize arabinose, rhamnose, galactose, sucrose, lactose, melibiose, cellobiose, raffinose, melizitose, inulin, salicin, pectin, inositol, and dulcitol at all. Ramamurthi (1957) reported that most species of the genus Corynebacterium produce only a slight amount of acid from carbohydrates. Dewolf in 1927 showed that C. diphtheriae metabolized glucose to yield formic, acetic, propionic, lactic and succinic acid and a small amount of ethyl alcohol (Jensen, 1952). According to Breed et al. (1957) and Carrier (1963), the plant pathogens are equally poor fermenters of carbohydrates. Most species, however, produce some acid, but no gas from glucose and maltose.

Nitrogen requirements. According to Mueller (1940), it is considered essential to have organic nitrogen in the medium for growth of the animal parasitic corynebacteria. Braun, Hofmeier and Mundal (1929) and Jensen (1952) reported that growth is better in media

containing peptone. Mueller (1940) has set out clearly the nitrogen requirements of C. diphtheriae. He found that aspartic acid or glutamic acid, and tryptophane are essential for growth. The more exacting strains may require as many as eight or more amino acids for growth (Mueller and Kapnick, 1935). According to Starr (1949), most of the plant pathogens need, in addition to the vitamins, casein hydrolysate. Vitamin requirements are varied. Many species need biotin, pantothenic acid, nicotinic acid and thiamin as vitamin supplements.

Proteolysis. Proteolysis is apparently slight or absent among the corynebacteria, with few exceptions (Carrier, 1963).

Presence of cytochrome oxidase and catalase. One of the most constant features of the genus Corynebacterium is the presence of cytochrome oxidase and catalase enzymes. Carrier (1963) investigated forty-four cultures of different species of corynebacteria and found all species except C. enzymicum possessed cytochrome oxidase and catalase. Most of the species were also found to possess oxidase.

Characteristics of the genus Lactobacillus

Morphology. The lactobacilli may vary in shape from short plump rods occurring singly, in pairs, in short chains, or in palisade arrangement as in some oral strains, to long slender rods occurring in pairs or in chains as seen in intestinal strains (Harrison and Opal, 1944). According to Harrison (1949), many cultures exhibit a typical diplobacillary form, often kidney-shaped. The lactobacilli are gram-positive, non-motile and non-spore forming organisms (Breed et al., 1957). They generally contain metachromatic granules (Thimann, 1963).

Conditions for growth. The lactobacilli are microaerophilic or anaerobic (Breed et al., 1957). They have varied nutritional requirements depending on the individual species. According to Pederson (1952), their nutritional requirements are complex, and most strains can not be cultivated on the usual nutrient media unless these are enriched by the addition of glucose or whey.

Some lactobacilli can grow with certain non-carbohydrate materials, e.g., citric acid as an energy source. Acetic acid also stimulates growth of many lactobacilli (Snell, 1952). Guirard et al. (1946) found that for other organisms, e.g., L. arabinosus, higher fatty acids and sterols partially replaced acetate.

Utilization of carbohydrates. The lactobacilli are as a group fermentative. The lactobacilli ferment glucose and similar aldehydic hexoses, carbohydrates which yield these simple sugars, and polyhydroxy alcohols (Breed et al., 1957). Glucose, lactose and sucrose are by far the most important compounds utilized as energy sources by the lactobacilli, glucose being the most commonly used carbohydrate for their culture (Snell, 1952). According to Snell (1952), the homofermentative organisms, e.g., L. caucasicus, L. casei, L. bulgaricus, L. plantarum, etc., ferment up to 95% of the utilized glucose, other hexose, or fermentable disaccharides to lactic acid. The remainder of the sugar is converted to carbon dioxide, traces of volatile acids, and cellular protoplasm. The lactic acid is formed by the reactions of the Embden-Meyerhof pathway. According to Breed et al. (1957), the type species, L. caucasicus, ferments glucose, sucrose, maltose and lactose but not starch. L. casei ferments glucose, fructose, mannose,

galactose, maltose, lactose, mannitol and salicin and sometimes sucrose. The lactic acid produced by L. caucasicus is mostly levo-rotatory, whereas the same produced by L. casei is mostly dextro-rotatory.

According to Pederson (1952), the heterofermentative species, e.g., L. pastorianus, L. buchneri, L. brevis, L. fermenti, etc., convert up to 50% of the glucose utilized to lactic acid, 20 to 25% to carbon dioxide and the rest to alcohol and acetic acid. Levulose is partly converted to mannitol, but, since mannitol is fermentable by many strains, it may be an intermediate rather than an end-product of fermentation.

Snell (1952) reported that both hetero- and homofermentative organisms characteristically ferment pentoses to acetic acid and lactic acid. Heath et al. (1956, 1958) showed that L. pentosus and L. plantarum produce equimolar amounts of acetic acid and lactic acid from pentoses.

Nitrogen requirements. Snell (1952) reported that the growth of lactobacilli is stimulated by the addition of ammonium salts to media that are deficient in certain amino acids. Most of the combined nitrogen and carbon that appears in the cell, however, must be supplied to these organisms as preformed organic compounds, which for one reason or another can not be synthesized by the cell.

According to Harrison (1942), the heterofermentative types require thiamin, whereas the homofermentative ones do not. Coolidge (1951) has shown that a change in cultivable strains from the hetero- to homofermentative type of mechanism is accompanied by a loss of thiamin

requirement. Snell (1952) reported that the biotin requirement of L. arabinosus is ten times as high in a medium lacking aspartic acid as in a medium containing ample amounts of this amino acid. Lardy et al. (1949) and Snell (1951) showed that this was because biotin was essential for synthesis of aspartic acid.

Presence of peroxidase. Walker and Kilgour (1965) demonstrated DPNH-peroxidase activity in cell-free extracts of L. casei. They described a method whereby they could isolate a partially purified preparation from the same source which contained both DPNH-oxidase and DPNH-peroxidase activities. However, they were unable to separate these two enzyme activities in their procedures.

Presence of cytochrome oxidase and catalase. One of the most unique and fundamental characteristics of the genus Lactobacillus is the lack of cytochrome oxidase and catalase (Thimann, 1963). All species, according to Breed et al. (1957), are catalase-negative. However, recently Whittenbury (1964) and Johnston and Delwiche (1965 a) demonstrated the existence of catalase in L. plantarum. Whittenbury (1964) reported three catalase-positive strains of L. plantarum which were found to be capable of incorporating hematin during growth to produce what appeared to be a typical heme-iron catalase, but Johnston and Delwiche (1965 a) noticed that when the organisms were grown in the absence of preformed hematin, they produced only the azide-insensitive enzyme. Johnston and Delwiche (1965 a) found that incubation of resting cells or cell-free extracts with hematin did not result in the production of active heme-enzyme, indicating that incorporation of hematin took place only when it was supplied during growth of the organisms. They reported that in some strains, grown in

the presence of the heme component, the non-heme catalase may coexist with a typical heme-iron catalase. They were also able to separate and characterize the two enzymes by appropriate fractionation procedure (Johnston and Delwiche, 1965 b).

Characteristics of the genus Microbacterium

Morphology. The microbacteria are all gram-positive, non-motile and non-spore forming (Breed et al., 1957). They are mostly wedge-shaped or cocco-bacillary rods (Orla-Jensen, 1919; Jensen, 1934; Doetsch and Pelczar, 1948). According to Doetsch and Pelczar (1948), the type species, M. lacticum, forms short, thin rods which are occasionally club-shaped. Angular and palisade arrangements are frequently noted in preparations made from cultures grown on solid media. The cells of M. flavum may be up to 0.9 μ. A characteristic attributed to this organism was that no cells were observed which were 10 μ long (Orla-Jensen, 1919; Witten, 1933). Some microbacteria, e.g., M. mesentericum form long, thin, filamentous cells (Witten, 1933). Doetsch and Pelczar (1948) reported that when microbacteria are grown under anaerobic conditions, their morphology is not significantly altered. However, the rod-shaped appearance of some cultures is less pronounced, and, in general, all are more coccoid in appearance. Metachromatic granules are observed when the cells are stained with Loeffler's methylene blue (Orla-Jensen, 1919). The granulation is more pronounced in cultures grown in milk. The microbacteria are not acid-fast by the Ziehl-Neelsen procedure. Electron micrographs reveal that their cell contents seem to be unusually dense (Doetsch and Pelczar, 1948).

Cultural characteristics. When grown in deep agar tubes, the microbacteria grow only in the upper part, and in stab cultures, they exhibit more or less pronounced surface growth (Orla-Jensen, 1919). The microbacteria form small (about 1.0 mm in diameter), smooth, round, convex, glistening white or gray surface colonies which are amorphous (Doetsch and Pelczar, 1948). M. flavum forms a finely flaked precipitate in broth medium (Orla-Jensen, 1919). M. lacticum produces pale greenish yellow pigment and M. flavum produces cream to canary-yellow pigment on agar slant (Breed et al., 1957). Orla-Jensen (1919) reported that in the case of M. flavum, the yellow pigment is more strongly apparent on sugar-containing media than sugar-free media.

Conditions for growth. The microbacteria are aerobic and facultative. Growth under anaerobic conditions is very slow, and pigment production is minimal (Doetsch and Pelczar, 1948). They grow best at 32 C (Breed et al., 1957), and poorly at temperatures above 35 C (Orla-Jensen, 1919).

Utilization of carbohydrates. The organisms of the genus Microbacterium present a physiological gradient with the saccharolytic acid-former, M. lacticum, at one extreme and the proteolytic gelatin-liquefier, M. liquefaciens, at the other extreme. Most species ferment glucose, galactose, fructose and mannose. Rhamnose, inulin, adonitol and sorbitol are not fermented (Doetsch and Pelczar, 1948). Orla-Jensen (1919) reported that the microbacteria never ferment pentoses. According to Doetsch and Pelczar (1948), some species are very active in attacking the widest range of carbohydrates with the production of considerable acid, while others, e.g., M. lacticum, are comparatively less active. M. lacticum ferments glucose, galactose, cellobiose,

maltose, lactose, trehalose, dextrin, starch and mannitol. M. flavum attacks only a few carbohydrates, e.g., glucose, mannitol and sometimes galactose and trehalose. None of the microbacteria produces gas from carbohydrates detectable by the usual culture technique. Speck (1943) reported that the microbacteria produce relatively small amounts of carbon dioxide from both milk and glucose broth in an atmosphere containing a limited oxygen supply. The acid produced by these organisms is predominantly lactic acid. Orla-Jensen (1919) reported that the microbacteria produce dextrorotatory (L+) lactic acid.

Nitrogen requirements. Breed et al. (1957) reported that media containing milk or yeast extract support good growth. Doetsch and Pelczar (1948) studied fourteen strains of M. lacticum and found that pantothenic acid is an absolute requirement for growth of these organisms. They devised a simple medium containing 1% glucose, 0.5% vitamin-free, salt-free casein hydrolysate, 0.025% l-asparagine, 0.01% dl-tryptophane, 0.001% l-cystine, 1 mg/ml d-calcium pantothenate and inorganic salts to grow these organisms. They found that omission of pantothenate from this medium results in a medium which fails to support growth of any of the strains tested by them. They found biotin and thiamin to be stimulatory.

Biochemical characteristics. The microbacteria are on the whole weak acid-formers and do not, for the most part, coagulate milk (Orla-Jensen, 1919). M. lacticum, however, occasionally acidifies and curdles milk. M. flavum produces no change in milk (Breed et al., 1957). One of the characteristic properties of M. flavum is that it

can tolerate 10% sodium chloride (Orla-Jensen, 1919). According to Doetsch and Pelczar (1948), the microbacteria do not produce indol and hydrogen sulfide. M. lacticum hydrolyzes starch and slightly reduces nitrate to nitrite. The microbacteria do not hydrolyze sodium hippurate. They are non-lipolytic, and, in general, non-proteolytic, since they neither liquefy gelatin nor digest the casein of milk.

Heat resistance. Heat resistance is used in Bergey's Manual (Breed et al., 1957) as a characteristic to describe the two recognized species of the genus Microbacterium. Skerman (1949) used this criterion as a distinguishing characteristic between the genus Microbacterium and the genus Corynebacterium. Speck (1943) suggested that heat resistance could be employed as a screening method in the isolation of members of the genus Microbacterium. Doetsch and Pelczar (1948) are of the opinion that thermal resistance does not appear to be a satisfactory characteristic for classification of the microbacteria. Although M. lacticum is reported to survive 85 C for $2\frac{1}{2}$ minutes in milk (Orla-Jensen, 1919), only three of the cultures tested by Doetsch and Pelczar (1948) were able to survive this treatment in yeast extract proteose peptone broth (pH 6.6). Doetsch and Pelczar (1948) concluded that milk itself must have enhanced the thermal resistance of the organisms. Rahn (1945) emphasized that this criterion should not be given too much importance in classifying microorganisms because it is associated with many other thermoduric organisms.

Presence of catalase. All members of the genus Microbacterium possess catalase (Orla-Jensen, 1919). Hansen (1938) reported that

hydrocyanic acid or iodoacetate inhibits the respiration of M. lacticum, indicating that the microbacteria differ from the true lactic acid bacteria not only in catalase content but also in their hemin content.

MATERIALS AND METHODS

Microorganisms

The original isolates of C. enzymicum which were described by Mellon (1917) and studied by Eberson (1918) are apparently no longer available. The two strains of C. enzymicum (8156 and 8155) employed in this study were isolated by Dr. L. H. Schwarz in 1940. No published report dealing with strain 8156 is available. Carrier (1963) studied strain 8155. These two strains of C. enzymicum (8156 and 8155) were obtained from the American Type Culture Collection. Three other species of Corynebacterium, one species of Microbacterium and one species of Lactobacillus were also employed in this study. C. diphtheriae (mitis) was supplied by Dr. Hauser (Director of Laboratories, Louisiana State Department of Health), C. kutscheri (11035), C. striatum (7940) and M. lacticum (8180) were obtained from the American Type Culture Collection. The culture of L. casei was obtained from Dr. A. R. Colmer.

Stock cultures were maintained in Brain Heart Infusion (BHI) semi-solid agar (0.3%) in screw-capped tubes. The cultures were stab inoculated. The corynebacteria were incubated at 37 C and the M. lacticum was incubated at 30 C for eighteen hours and stored in the refrigerator at 4 C. Transfers were made at intervals of approximately two months. The culture of L. casei was maintained in litmus milk at 30 C and transferred every week.

Media

The following media were used in this study and prepared as indicated. Sterilization, unless otherwise indicated, was accomplished in the autoclave at 121 C for 15 minutes.

Tryptone broth

Bacto-tryptone	1.0 g
Sodium chloride	0.5 g
Dipotassium phosphate	0.1 g
Distilled water to	100 ml
pH	7.0

Casamino acids medium

Bacto-casamino acids	0.3 g
Bacto-yeast extract	0.1 g
Distilled water to	100 ml
pH	7.0

Tryptone-glucose-yeast extract (TGY) broth

Bacto-tryptone	1.0 g
Bacto-yeast extract	1.0 g
Dipotassium phosphate	0.26 g
Bacto-dextrose	0.5 g
Distilled water to	100 ml
pH	7.0

Synthetic medium (Braun and Hofmeier, 1927)

Na_2SO_4	0.5 g
$MgSO_4 \cdot 7H_2O$	0.005 g
KH_2PO_4	0.05 g
K_2HPO_4	0.15 g
Na aspartate	0.5 g
Cystine	0.0125 g
$CH_3COONa \cdot 3H_2O$	0.5 g
Distilled water to	100 ml
pH	7.0

Catalase test medium (Johnston and Delwiche, 1965 a) Basal medium

Bacto-peptone	0.5 g
Bacto-yeast extract	0.5 g
$MnSO_4 \cdot 4H_2O$	0.05 g
Tween 80	0.05 g
Sodium citrate	0.5 g
Bacto-dextrose	0.5 g
Distilled water to	100 ml
pH	6.8

Bovine blood was collected aseptically and was first lysed by the addition of an equal volume of sterile distilled water and then boiled for 15 minutes to destroy the blood catalase. The blood, as a substitute for hematin, was added to give a final concentration of 5%.

Additional media were employed as indicated in the following sections. All were Difco products unless otherwise indicated.

Attempts to isolate C. enzymicum

Mellon (1916) originally isolated C. enzymicum from the lungs of a patient. He reported that this organism acidified and coagulated milk. This observation of Mellon and the similarity of the organism to lactobacilli suggested the possibility that C. enzymicum might occur in raw milk or in the human mouth. Therefore some efforts were made to isolate the organism from these sources.

Two hundred and thirty-five milk samples from individual cows of the Louisiana State University dairy and Borden's dairy herds (Baton Rouge) were collected aseptically in sterile test tubes. The samples were kept overnight at room temperature and then stored in the refrigerator until plated. Teeth scrapings from 22 patients suffering from dental caries were collected by Dr. Hoover, a practicing dentist in Baton Rouge, and placed in 6 ml of BHI broth. Throat swabs were taken from 31 students and placed in 6 ml of BHI broth.

Media for isolation were made somewhat selective by adding 1 ml of filter-sterilized 1% potassium tellurite to 99 ml of sterile Tinsdale Base and BHI agar at 45 C. The final concentration of tellurite in the media was therefore 0.01%. After mixing thoroughly, the media were poured into sterile Petri dishes and allowed to harden. For the isolation of C. enzymicum, a loopful of each sample was streaked on the plates and incubated at 35 C with frequent observations for 15 days. Colonies were examined using a dissecting microscope and smears from colonies which were similar to colonies of C. emzymicum were stained by Gram's method.

Morphological studies

Stained smears of the organisms were examined for their characteristic form and arrangements of cells. The presence or absence of motility was determined by growing the organisms in SIM medium. A 1% aqueous solution of nigrosin was used to demonstrate capsules. Acid-fastness was determined by the Ziehl-Neelsen method. Cells were stained by the Schaeffer-Fulton method and observed for spores. An alcoholic solution of methylene blue containing 0.3 g methylene blue, 30 ml 95% ethanol and 100 ml distilled water was used to demonstrate metachromatic granules. Smears were stained for 20 seconds and examined under the microscope.

Physiological and biochemical studies

The effect of temperature on growth. A loopful of culture was inoculated into test tubes containing 10 ml of tryptone broth supplemented with 0.5% glucose. The effect of temperature on growth was determined by incubating the cultures in thermostatically controlled water baths at 4, 20, 25, 28, 29, 30, 33, 35, 37, 39, 40 and 45 C for 12 hours and recording the optical density at 600 mμ with a spectrophotometer (Bausch and Lomb Spectronic 20).

The effect of pH on growth. The organisms were inoculated into test tubes containing 10 ml of tryptone broth at pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 9.5 and incubated at 33 C for 24 hours. The pH was adjusted with N HCl and N NaOH. Growth was observed by measuring the optical density at 600 mμ with a spectrophotometer (Bausch and Lomb Spectronic 20).

The effect of oxygen on growth. The organisms were stab inoculated into BHI semi-solid agar (0.3%) medium tubed in deep columns (8 cm) and observations for growth were made after 72 hours incubation at 33 C.

The effect of free oxygen on the growth of the organisms was also determined by growing them in nutrient broth with and without glucose (0.5%) using both stationary and aerobic shake culture (on a rotary shaker, New Brunswick Scientific Company) techniques. The organisms were inoculated (at the rate of 0.5% inoculum) into 100 ml of media in 250 ml Erlenmeyer flasks and incubated at 33 C for 24 hours. Aliquots were removed at 0, 5, 7, 9, 11 and 24 hours and the pH and optical density (at 600 mμ) recorded.

Heat resistance. Tubes containing 10 ml of tryptone-glucose (0.5%) broth were placed in thermostatically controlled water baths at 50, 55, 60, 65, 70 and 75 C for 30 minutes. Heat resistance was determined by inoculating the tubes with a loopful of culture and subjecting the tubes to 10 minutes exposure at 50 and 55 C and to a 3 minutes exposure at 60, 65, 70 and 75 C in thermostatically controlled water baths. The tubes were immediately cooled and incubated at 33 C for 72 hours before final observations for growth were made.

Sodium chloride tolerance. The organisms were inoculated into test tubes containing 10 ml of tryptone-glucose (0.5%) broth and incubated at 33 C for 7 days. Observations for growth were made visually every day.

Growth in simple media. The organisms were inoculated separately into 100 ml of the Braun and Hofmeier's synthetic medium and casamino acids medium (with and without 0.5% glucose) in 250 ml Erlenmeyer

flasks and incubated at 33 C for 36 hours. Observations for growth were made at 0, 8, 16, 24 and 36 hours. Subcultures were made in Braun and Hofmeier's medium to insure that growth in this medium was not due to nutrients in the inoculum.

Utilization of carbon and nitrogen compounds. The basal media used were Braun and Hofmeier's synthetic medium and tryptone (1%) broth. The following organic acids (0.5%) were added separately to the basal media: pyruvate, acetate, citrate, isocitrate, α-ketoglutarate, succinate, fumarate, malate and oxaloacetate.

The following nitrogen compounds (Friedman and Roessler, 1961) were added separately to the basal media: l-histidine-HCl, l-cysteine-HCl, l-leucine, l-phenylalanine, l-proline, l-threonine, l-tyrosine and glycine (each 0.1%), l-arginine-HCl, l-lysine-HCl, dl-isoleucine, dl-methionine, dl-valine, dl-tryptophane, dl-serine (each 0.2%), l-asparagine, casein hydrolysate (each 0.5%), l-glutamine (0.6%), dl-alanine, dl-aspartate, l-glutamate and B-alanine (each 0.8%).

The following amino acids mixture of Friedman and Roessler (1961) was also added to each of the two basal media: l-cysteine, l-leucine, l-histidine (each 0.01%), l-arginine, dl-isoleucine, dl-valine, dl-methionine, dl-tryptophane (each 0.02%) and l-glutamine (0.06%).

The organisms were inoculated into tubes containing 10 ml of the media (pH adjusted to 7.0) and incubated at 33 C for 72 hours and observed for growth. Observations were made to determine whether or not Braun and Hofmeier's synthetic medium supplemented with the compounds would support growth of the organism, and whether or not tryptone broth containing the carbon and nitrogen compounds would show an enhancement of growth.

The effect of thiamin on growth and pigment production. The organisms were inoculated separately into 50 ml of Braun and Hofmeier's synthetic medium and tryptone broth, each containing 50 µg/ml thiamin and incubated at 33 C for 72 hours. Observations were made visually for growth and pigment production.

Oxidative and fermentative utilization of carbohydrates. Different carbohydrates were filter-sterilized and added separately at the rate of 1.0% to 16 x 150 mm tubes containing 10 ml of sterile O.F. medium (Difco) (final pH 7.0) at 45 C and mixed. The tubes were allowed to harden and then inoculated in duplicate by stabbing to the bottom of the medium. After inoculation approximately 2 ml of melted sterile 3% agar was pipetted on to the top of the agar of one inoculated tube. All tubes were incubated at 33 C for two weeks. Recordings of growth were made at intervals. Tubes which turned yellow in the upper third were recorded as "oxidative"; if yellow in the lower half the test was recorded as "fermentative". If after two weeks no color change was observed, it was concluded that the carbohydrate was not attacked.

Tests for various biochemical properties. The organisms were tested for their action in litmus milk, indol and hydrogen sulfide production, reduction of nitrate and methylene blue, methyl red and Voges-Proskauer reactions and ability to hydrolyze gelatin, starch and urea according to the standard bacteriological techniques described in the Manual of Microbiological Methods (1957). The ability to hydrolyze sodium hippurate was determined by the method of Ayers and Rupp (1922). The ability to hydrolyze esculin was tested by growing the

organisms for 5 days at 33 C in BHI agar containing 0.1% esculin. Hydrolysis was determined by the development of a black color upon the addition of 0.5 ml of a 5% solution of ferric ammonium citrate on the colonies. Ability to grow in the presence of KCN was determined by inoculating the organisms into KCN broth base (Difco) supplemented with 0.0075% KCN and incubating at 33 C for 72 hours. To test for the reduction of tetrazolium salts, the cultures were grown for 24 hours on BHI agar plates. The reducing properties of the colonies were tested by means of the following reagents: triphenyl tetrazolium, nitroblue tetrazolium, blue tetrazolium, neo-tetrazolium, iodonitro tetrazolium and tetranitro blue tetrazolium (all 0.5%). Tellurite reduction was demonstrated by growing the organisms for 24 to 48 hours at 33 C on BHI agar containing 0.1% filter-sterilized potassium tellurite.

Test for DNase activity. The DNase agar (Difco) plates were heavily inoculated in a band across the center of the plates. After 48 hours incubation at 33 C the plates were flooded with a N HCl solution. DNase activity was indicated by a clear zone around the band of growth.

Tests for oxidase and cytochrome oxidase activities. BHI agar plates were streaked with the cultures and incubated at 33 C for 72 hours. Oxidase activity was determined by flooding the plates with a freshly prepared 1% aqueous solution of N, N-dimethyl-p-phenylenediamine monohydrochloride. A positive test was indicated by a color change of the colonies through pink to black.

To test for cytochrome oxidase, the modified benzidine test was used. The plates were flooded with the benzidine dihydrochloride

reagent (Deibel and Evans, 1960), followed by an equal volume of 5% hydrogen peroxide. A blue-green to deep blue coloration of the colonies indicated the presence of iron-porphyrin compounds.

Test for catalase activity. As a screening method BHI agar plates were streaked with the cultures and incubated at 33 C for 72 hours. One drop of a 1% solution of hydrogen peroxide was then put on the isolated colonies and observed for the evolution of gaseous oxygen.

The organisms were also examined for the presence of catalase by the method of Johnston and Delwiche (1965 a). Cells were grown in 500 ml quantities of catalase medium (see Media) in two 1-liter Erlenmeyer flasks on a rotary shaker (New Brunswick Scientific Company) at 35 C for 48 hours. The cells were then centrifuged down, washed with distilled water and suspended in 10 ml of 0.1 M potassium phosphate buffer (pH 6.9). Cell-free extracts were prepared by sonicating the suspension for 5 minutes at 20 kc with a Branson Sonifier (0 C) and removing the cellular debries by centrifugation. In the catalase assay procedure, residual peroxide was determined by the iodometric method of Herbert (1955). The assay system in serological tubes, consisted of 1.0 ml of 0.1 M phosphate buffer (pH 6.5), 0.3 ml of 0.01 M sodium azide (when included), and 0.5 ml of the cell suspension or cell-free extract, the total volume was made up to 2.5 ml with distilled water. The reaction was initiated by the addition of 0.5 ml of 0.01 M H_2O_2 solution and terminated by the addition of 2.0 ml of N H_2SO_4. In the control tube, the initial H_2O_2 concentration was obtained by adding the H_2SO_4 before the enzyme. The reaction in each tube was stopped after 15, 30, 45 and 60 seconds. The acidified

reaction mixture was then assayed directly for residual peroxide by the iodometric procedure. The H_2O_2 remaining in each tube was determined by adding 0.5 ml of 10% KI, 1 drop of 1% ammonium molybdate, and after the tube has stood for 3 minutes, titrating the liberated iodine with a 0.01 N sodium thiosulfate (starch-iodide indicator), the whole content of each tube being titrated in situ.

Test for peroxidase activity. The method described by Walker and Kilgour (1965) was used for the determination of peroxidase.

The organisms were grown in TGY broth at 35 C for 20 hours using aerobic shake culture technique. The cells were harvested from 3 liters of culture medium by centrifugation and suspended in 10 ml of 0.02 M phosphate buffer (pH 7.0). The suspension was then sonicated for 5 minutes at 20 kc with a Branson Sonifier at 0 C. The material was centrifuged at 28,000 x g for 30 minutes at 1 C and the supernatant was collected.

For the assay of the enzyme, 40 μmoles of acetate buffer (pH 5.4), 0.13 μmole of DPNH and 0.9 μmole of H_2O_2 and enzyme to final volume of 3.0 ml was used. The measurement was carried out in 3.0 ml Beckman DB cuvette under aerobic conditions and also in 3.0 ml silica cells fused to Thunberg tubes (Pyrocell Manufacturing Corporation) under anaerobic conditions by evacuating the cells and introducing nitrogen gas. Changes in optical density were measured at 340 mμ with a Beckman DB spectrophotometer equipped with a recorder.

Utilization of pentoses. Ribose, xylose and arabinose were filter-sterilized and added separately at the rate of 0.25% to tryptone (1%) broth. The organisms were inoculated into 250 ml of media and

incubated at 33 C for 24 hours. Aliquots were removed at 0, 8, 16 and 24 hours and the pH and optical density (600 mμ) were recorded.

Ribose dissimilation was determined by inoculating the organisms into 250 ml of tryptone broth containing 0.22% filter-sterilized ribose and incubating the cultures at 33 C for 36 hours. Aliquots were removed at 0, 12, 24 and 36 hours and the presence of ribose and lactic acid were quantitatively measured. Optical density (600 mμ) and pH were also recorded. The amount of ribose and lactic acid initially present in the sugar-free medium was determined for correction.

Ribose was determined by the quantitative orcinol method described by Clark (1964). The culture supernatant to be used for ribose determination was first deproteinized by the method of Nelson (1944). One volume of culture supernatant was mixed with 15 volumes of distilled water. Then 2 volumes of 0.3N barium hydroxide were added and mixed. After a few minutes it was filtered and 3 ml of this filtrate was used for the determination. In addition, a blank containing 3 ml of distilled water was prepared. To each tube was added 6 ml of the orcinol acid reagent and 0.4 ml of 6% alcoholic orcinol. All tubes were heated in a boiling water bath for 20 minutes. The tubes were cooled and the optical density of each was measured at 660 mμ with a spectrophotometer (Bausch and Lomb Spectronic 20) using a red filter. A reagent blank was used for zero adjustment. A ribose standard curve was prepared simultaneously, using 3, 5, 10, 15, 20 and 30 μg/ml of ribose. The values for the unknown were obtained from the standard curve and recorded after multiplying by the dilution factor.

Lactic acid was determined colorimetrically by the method of Neish (1952). Different dilutions of culture supernatant were prepared.

One ml of the diluted culture supernatant was pipetted into a test tube and 0.05 ml of the 4% copper sulfate solution was added. Six ml of concentrated sulfuric acid was pipetted in using a propipette. The tubes were allowed to stand 5 minutes in the air, and then cooled below 20 C in an ice-water bath. Five-hundredth ml of 1% p-hydroxy-diphenyl reagent was added without touching the sides of the tubes, mixed in thoroughly and allowed to stand 6 to 8 hours at room temperature. At the end of this period the optical density was measured at 570 mμ with a spectrophotometer (Bausch and Lomb Spectronic 20). A reagent blank and a standard curve were run with the determination of the unknown. The standard was prepared by accurate dilutions of the standard lithium lactate solution (1, 3, 5 and 10 μg/ml). The values of lactic acid for the unknown were obtained from the standard curve and recorded after multiplying by the dilution factor.

An experiment was carried out to determine the ribose utilization by the organism in the presence of glucose. For this two different concentrations of filter-sterilized glucose (0.07% and 0.16%) were added along with 0.22% ribose in tryptone broth. Cultures were inoculated as described in the previous experiment. Aliquots were removed at 0, 12, 24 and 36 hours and this time glucose was estimated along with ribose and lactic acid. Optical density (600 mμ) and pH were also recorded.

Glucose was determined by the modified Somogyi method of Nelson (1944). The culture supernatant was first deproteinized by the method already described. To a 1 ml portion of deproteinized filtrate in a test tube was added 1 ml of a mixture of copper reagents A and B

(25:1 v/v), and the mixture was heated for 20 minutes in a boiling water bath. The tubes were then cooled in a can of cold water. One ml of arsenomolybdate reagent was added and thoroughly mixed. A green color developed which could be diluted and the optical density finally read at 500 mμ with a spectrophotometer (Bausch and Lomb Spectronic 20) using a reagent blank for zero adjustment. A glucose standard curve was prepared simultaneously using 1.5, 3, 5, 15 and 30 μg/ml glucose. The values for the unknown were obtained from the standard curve and recorded after multiplying by the dilution factor.

Utilization of glucose. The organisms were inoculated (5% inoculum) into 500 ml of tryptone broth containing 0.5% filter-sterilized glucose in 1-liter Erlenmeyer flasks and incubated at 33 C for 48 hours. Aliquots were removed at 0, 8, 12, 24, 36 and 48 hours and the pH, optical density (600 mμ), titratable acidity, volatile acidity and lactic acid were recorded.

Titratable acidity was determined by boiling 5 ml of culture supernatant for a few minutes to drive off the carbon dioxide and then titrating against 0.1N NaOH with phenolphthalein indicator.

Volatile acidity was determined by the method of Neish (1952). Ten ml of culture supernatant was adjusted to below pH 2.0 with N H_2SO_4. This was then steam-distilled for exactly 10 minutes. The distillate was collected and titrated against 0.1N NaOH with phenolphthalein indicator.

Glucose dissimilation was studied by inoculating the organisms into 250 ml of tryptone broth supplemented with 0.25% filter-sterilized glucose in 500 ml Erlenmeyer flasks. The cultures were

incubated at 33 C for 36 hours. Aliquots were removed at 0, 8, 16, 24 and 36 hours and the presence of glucose and lactic acid was quantitatively estimated according to the methods previously described. The values for the unknown were corrected by determining the amount of glucose and lactic acid initially present in the glucose-free medium. Optical density (600 mμ) and pH were also recorded.

The optical activity of lactic acid. The organisms were inoculated into 250 ml of medium (containing 1% tryptone, 1% yeast extract and 0.25% filter-sterilized glucose) in 500-ml Erlenmeyer flasks and incubated at 33 C for 36 hours. At the end of this period the total lactic acid present in the culture was determined by the method of Neish (1952).

The optical activity of the lactic acid produced from glucose was determined by the method described by Cato and Moore (1965). One ml of culture supernatant was deproteinized with 1 ml of 3.5% perchloric acid. After 5 minutes at room temperature, the sample was clarified by centrifugation for 10 minutes at 2000 x g. A 0.1 ml sample of the supernatant liquid was pipetted into a 12 x 75 mm test tube. To this was added 3 ml buffer (0.5 M glycine, 0.4 M hydrazine sulfate dissolved in redistilled water; pH adjusted to 9.0 with 2N NaOH), 0.03 ml L(+) lactic dehydrogenase (LDH) (Nutritional Biochemical Corporation; 41 mg protein per ml, diluted with an equal volume of redistilled water) and 0.2 ml nicotinamide adenine dinucleotide (NAD) solution (Nutritional Biochemical Corporation; assay 98%; 20 mg per ml redistilled water). Both the LDH and NAD were freshly prepared and kept in an ice-water bath during preparation of the tubes. For the standard, a

sample of L(+) lactic acid sodium (d-lactic acid sodium; 40% sol., M.A.) was diluted to contain 300 mg of lactic acid per ml. The concentration of this standard was determined by the method of Neish (1952). A series of standards was prepared with a set of unknown samples from 2 μmoles L(+)-lactate per 0.1 sterile medium (tryptone yeast extract broth containing 0.25% glucose treated with 3.5% perchloric acid v/v). Dilutions were made with lactate-free medium similarly treated to give 0.1, 0.2, 0.4, 0.5, 0.8 and 1.0 μmole lactic acid per 0.1 ml solution. All tubes were incubated 60 minutes at 30 C in a thermostatically controlled water bath. Each test solution was poured into a colorimeter tube and its absorbance read at 366 mμ with a spectrophotometer (Bausch and Lomb Spectronic 20) against a reagent blank prepared and treated exactly as the standards but without the lactate substrate. The blank was not stable and was used only once to bring the instrument to zero reading. The reading of an unmatched tube containing medium and water was then used to restandardize the instrument periodically. The same colorimeter tube used for the unstable blank was used throughout the series. The amount of L(+)-lactate per ml of culture was determined by multiplying μmoles of lactate estimated from comparison with the standard curve by the sample dilution factor, 20.

Test for pathogenicity

Mellon (1917) reported that C. enzymicum is pathogenic to rabbits, mice and guinea pigs. Since Mellon's isolates are no longer available, it was considered desirable to test a strain of C. enzymicum (8156) obtained from the American Type Culture Collection.

The organisms were grown in BHI broth to their logarithmic phase of growth cycle (5 hours). Three rabbits were selected for the test, one for the inoculation by the subcutaneous route, the other by the intraperitoneal route and the third served as a control being inoculated with sterile physiological saline solution. A 0.1 ml inoculum was used and was introduced with a 23 gauge hypodermic needle according to the standard technique described in the Manual of Microbiological Methods (1957). The animals were inspected daily for three weeks to determine death or to ascertain the first appearance of any clinical symptoms.

Characterization of DNA by base composition

Isolation of DNA. DNA was isolated by the method of Marmur (1961) with some modifications.

A 5-liter culture of C. enzymicum (8156) was grown at 33 C to the logarithmic phase (about 5 hours) of the growth cycle. The cells were then sedimented by centrifugation at 0 C and resuspended in 50 ml of cold BPES (0.006 M Na_2HPO_4, 0.002 M NaH_2PO_4, 0.001 M disodium EDTA and 0.18 M NaCl, adjusted to pH 9.0 with NaOH) buffer. The cell suspension was then heated for 20 minutes at 70 C to inactivate nucleases (Massie and Zimm, 1965). Lysozyme was added to 1 mg/ml and the mixture was then incubated at 37 C in a water bath for 30 minutes with occasional shaking. Lysis was effected by the addition of 2.0 ml of 25% sodium lauryl sulfate and the mixture was placed in a 37 C water bath for 10 minutes. Lysis of the culture resulted in some clearing and a dramatic increase in viscosity due to the release of the nucleic acid components. (Step 1.)

To the lysed suspension, sodium perchlorate (5 M) was added to a final concentration of 1 M (Step 2). The mixture was shaken with an equal volume of chloroform-isoamyl alcohol (24:1 v/v) in a ground glass stoppered flask for 30 minutes. The resulting emulsion was separated into 3 layers by a 10 minute centrifugation (0 C) at 8000 r.p.m. in a Sorvall RC-2. The upper aqueous phase contained the nucleic acids and was carefully pipetted off into a tube (Step 3). The nucleic acids were precipitated by gently layering approximately 2 volumes of 95% ethanol on the aqueous phase. The layers were then gently mixed with a stirring rod. The nucleic acids "spooled" on the rod as a thread-like precipitate and were removed after draining free of excess alcohol by pressing the spooled rod against the tube. The precipitate was then transferred to 15 ml of dilute saline-citrate (0.015 M NaCl plus 0.0015 M trisodium citrate) and gently removed from the stirring rod by swirling it back and forth. The solution was then adjusted approximately to standard saline-citrate (0.15 M NaCl plus 0.015 M trisodium citrate) concentration by adding concentrated saline-citrate (1.5 M NaCl plus 0.15 M trisodium citrate) (Step 4). Deproteinization (Step 3) was repeated about 6 to 8 times until very little denatured protein was visible at the interface. The supernatant obtained after the last treatment in the series of deproteinization steps, was precipitated with ethanol and dispersed in saline citrate (about 0.75 the supernatant volume) in the manner already described. Ribonuclease (Nutritional Biochemical Corporation) was added to a final concentration of 50 μg/ml, and the mixture incubated for 30 minutes at 37 C (Step 5). Following the digestion of the RNA, the mixture was again

subjected to deproteinization (Step 3) 3 to 4 times to get rid of the last traces of protein. The supernatant, after the last such treatment, was precipitated with ethanol and the DNA threads which were collected on the glass rod were drained and dried in a vacuum desiccator (40 C) and finally stored in the refrigerator (Step 6).

Flow sheet of procedure for the isolation of DNA of C. enzymicum.

Cell suspension

(in 50 ml BPES buffer at pH 9.0)

↓

Heated for 20 min at 70 C

↓

Lysozyme added to 1 mg/ml at 37 C for 30 min

↓

2.0 ml of 25% Na lauryl sulfate added at 37 C for 10 min

↓

Lysis occurred (Step 1)

↓

5M Na perchlorate added to final conc. of 1M (Step 2)

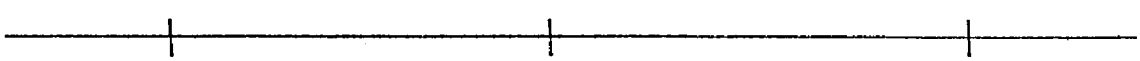
↓

Shaken with equal vol of chloroform-isoamyl alcohol for 30 min

↓

Centrifuged at 8000 r.p.m. for 10 min

↓



Flow sheet of procedure for the isolation of DNA of C. enzymicum (Continued)

Upper aqueous phase	Interface	Lower chloroform layer
↓	↓	↓
(Nucleic acids, removed) (Step 3)	(denatured protein, discarded)	(discarded)

↓

Precipitated with 2 vol of 95% ethanol

↓

Ppt. dissolved in 15 ml saline-citrate (final conc. 0.15 M NaCl + 0.015 M trisodium citrate) (Step 4)

↓

Step 3 repeated six to eight times with 15 min shaking

↓

Step 4 repeated

↓

Ribonuclease added to 50 μg/ml at 37 C for 30 min (Step 5)

↓

Step 3 repeated 3 to 4 times

↓

Precipitated with 2 vol of 95% ethanol

↓

DNA preparation dried in vacuum desiccator (40 C) and stored in the refrigerator (Step 6).

Hydrolysis of DNA. Hydrolysis of DNA was carried out by Bendich's (1957 a) method with a slight modification.

The DNA preparation (10.65 mg) was placed in a Pyrex glass tube, 0.5 ml of 98% formic acid added, and the tube sealed with an oxygen gas flame. The sealed tube was placed in a 185 C hot air oven for 90 minutes. After cooling, the tube was opened cautiously (since pressure developed owing to the formation of carbon monoxide resulting from the decomposition of formic acid at elevated temperature) and the contents were evaporated to dryness in a vacuum desiccator at 40 C under reduced pressure. N HCl was added to a final concentration of 3 to 4%. The organic bases were allowed to dissolve and the material centrifuged. The upper yellowish layer containing the dissolved bases was removed.

Paper chromatography. The technique of Bendich (1957 b) was followed. A portion (about 20 µl) of the hydrolystate was applied along with base standards (obtained from Nutritional Biochemical Corporation) to sheets of Whatman No. 1 filter paper (20 x 50 cm). Seven longitudinal lines (2.5 cm apart) were drawn in pencil to divide the paper sheet into 8 equal lanes. The hydrolysate was deposited in the center of two of the lanes on a transverse "starting line" drawn across the lanes about 8 cm from the top of the sheet. An identical volume of N HCl was deposited in a third lane (between the unknowns) to serve as a blank. In the remaining lanes were placed about 10 µl (approximately 25 µg) of each of the bases made up in N HCl. The spots were dried with a hair drier in a stream of warm air.

The chromatograms were placed in a glass cylindrical jar fitted with a glass trough supported about 10 cm from the top. The

isopropanol-12N HCl-H_2O (65-16.7-18.3 v/v) solvent system was placed in the trough and some on the bottom of the jar to equilibrate the chamber. The chromatograms were placed in the jar and the bases contained therein were allowed to separate by the solvent system using descending chromatography until the solvent front had moved about 40 cm from the starting line at room temperature. At the end of this period, the chromatograms were hung upside down and dried in a hood in an atmosphere of ammonium hydroxide to neutralize the HCl.

The individual spots of the base standards and those of the separated bases of the DNA hydrolysate were located on the chromatograms as dark patches against a background of general fluorescence when viewed in a dark room under long wave UV radiation. The spots were outlined in pencil and rectangles containing them were cut out. Identical areas of paper were cut from corresponding portions in the blank lane. Contact with fingers was avoided. Each area of paper was then cut up in small pieces and placed in test tubes and eluted in the closed tubes with constant shaking for 2 hours at room temperature with 5 ml of 0.1 N HCl. The eluates were centrifuged and supernatant collected.

Quantitation. The amount of each base was estimated by the differential spectrophotometric technique of Bendich (1957 b). The concentrations of the individual bases were measured with a Beckman DB spectrophotometer by calculating the difference in the extinction values (ΔE) read at the wave length of maximum absorption and at another wave length, such as 290 or 300.

For calculation of the concentrations of the bases in the unknown (DNA hydrolysate), the values obtained by Bendich (1957 b) under

standard conditions were used as reference values. Bendich found the following values by measuring standard solutions containing 10 μg of base per ml of 0.1 N HCl:

Adenine:	$E_{262.5} = 0.930$	Guanine:	$E_{249.0} = 0.737$
	$E_{290.0} = 0.030$		$E_{290.0} = 0.262$
	$\Delta E = 0.900$		$\Delta E = 0.475$
Thymine:	$E_{265.0} = 0.632$	Cytosine:	$E_{276.0} = 0.910$
	$E_{290.0} = 0.083$		$E_{300.0} = 0.047$
	$\Delta E = 0.549$		$\Delta E = 0.863$

The extinction values for an unknown base sample (ΔEx) were read at the respective wave length and ΔE values were referred from the above table (Bendich, 1957 b). Since Bendich used 10 μg of each base in 1 ml, therefore there were 10 x $\Delta Ex/\Delta E$ μg of base. The total eluate (5.0 ml) thus contained 5 times the amount of base calculated in 1 ml sample. The percentage of each base was calculated by dividing the amount of each individual base by the total μg of all bases in the sample.

RESULTS

Attempts to isolate C. enzymicum

Most of the colonies isolated from milk samples, throat swabs and teeth scrapings of patients suffering from dental caries appeared to be streptococci, staphylococci or lactobacilli. Only a few suspicious appearing colonies were found and were examined for their morphological, cultural and physiological characteristics and compared with those of C. enzymicum. None of these, however, were found to resemble C. enzymicum.

Morphological characteristics

The typical angular and palisade arrangements characteristic of the genus Corynebacterium were not observed in the case of C. enzymicum. The organisms appeared as very short coccoid rods occurring mostly in small clumps or pairs but very seldom in tetrads or in short chains. Unlike Carrier's (1963) observation, the organisms were found to stain readily with methylene blue and metachromatic granules were visible when the cells were taken from Loeffler's blood serum. Both strains of C. enzymicum studied presented a morphological picture similar to that described by Carrier (1963).

Physiological and biochemical characteristics

The effect of temperature on growth. The organisms did not grow at 4 C and 45 C and poorly at room temperature (25 C). Breed et al. (1957) and Carrier (1963) reported that the optimum temperature for growth of this species was 37 C, but in this study the optimum

temperature appeared to be between 30 and 33 C (Tables 1, 2 and Figures 1, 2).

The effect of pH on growth. The optimum pH for growth of C. enzymicum was found to be near 7.0. There was no growth at pH 4.0 and very slight growth at pH 9.0 (Table 3 and Figure 3).

The effect of oxygen on growth. The organisms were found to be markedly indifferent to oxygen. No difference in growth was observed when the organisms were grown under stationary and aerobic shake culture conditions (Table 4 and Figure 4). In the stab culture, the organisms did not grow on the surface but grew along the stab to the bottom. In Warburg vessels there was no oxygen uptake.

Heat resistance. The thermal death time of this organism in tryptone-glucose broth was found to be 60 C for 3 minutes (Table 5).

Sodium chloride tolerance. C. enzymicum did not grow at a sodium chloride concentration of 6.5% or above. Growth occurred at a concentration of 6.0% sodium chloride (Table 6).

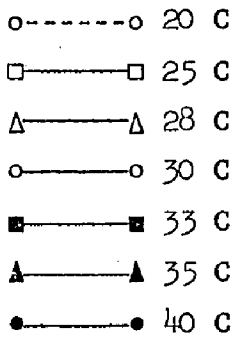
Growth in simple media. Braun and Hofmeier's (1927) synthetic medium did not support the growth of C. enzymicum. The organisms grew very little in ordinary media, such as nutrient broth (Table 4 and Figure 4), casamino acids medium (Table 7 and Figure 5) or tryptone broth without added sugar. Media containing fermentable sugar markedly enhanced growth. In such media the organisms grew luxuriantly producing considerable acid but no detectable gas.

Utilization of carbon and nitrogen compounds. The organisms were unable to utilize pyruvate, acetate and any intermediate of the tricarboxylic acid cycle, indicating that they lacked enzymes

Table 1. The effect of temperature on the growth of C. enzymicum (8155) in tryptone-glucose broth.

Temperature	Incubation Time (hrs)				
C	4	6	8	10	12
	Optical density at 600 mμ				
4	0.00	0.00	0.00	0.00	0.00
20	0.00	0.00	0.00	0.03	0.07
25	0.00	0.00	0.02	0.10	0.15
28	0.00	0.01	0.10	0.18	0.22
29	0.00	0.03	0.22	0.24	0.25
30	0.00	0.07	0.51	0.76	0.77
33	0.00	0.14	0.72	0.74	0.73
35	0.00	0.13	0.68	0.70	0.70
39	0.00	0.05	0.32	0.59	0.59
40	0.00	0.02	0.30	0.40	0.40
45	0.00	0.00	0.00	0.00	0.00

Figure 1. The effect of temperature on the growth of C. enzymicum (8155) in tryptone-glucose broth. Data from Table 1.



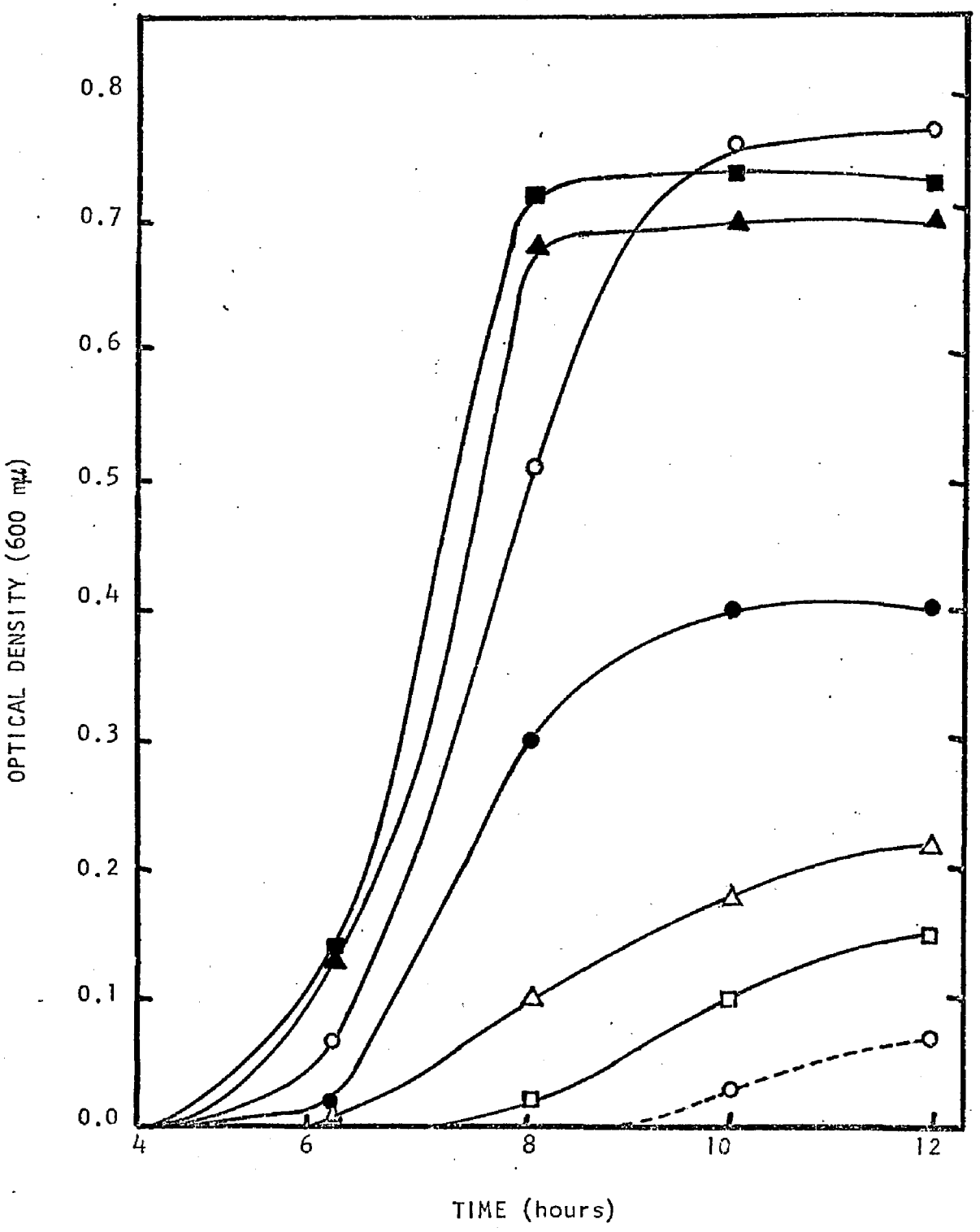
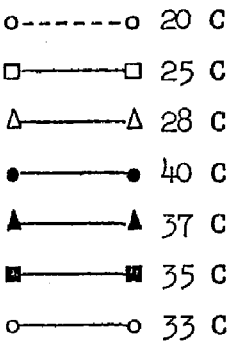

OPTICAL DENSITY (600 mμ)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
4
6
8
10
12
TIME (hours)

Table 2. The effect of temperature on the growth of C. enzymicum (8156) in tryptone-glucose broth.

Temperature	Incubation Time (hrs)				
C	4	6	8	10	12
	Optical density at 600 mμ				
4	0.00	0.00	0.00	0.00	0.00
20	0.00	0.00	0.02	0.06	0.09
25	0.00	0.00	0.06	0.15	0.22
28	0.00	0.05	0.14	0.25	0.32
29	0.00	0.07	0.28	0.35	0.35
33	0.00	0.32	0.74	0.76	0.76
35	0.00	0.30	0.67	0.68	0.68
37	0.00	0.25	0.63	0.64	0.64
39	0.00	0.12	0.45	0.53	0.55
40	0.00	0.02	0.32	0.41	0.42
45	0.00	0.00	0.00	0.00	0.00

Figure 2. The effect of temperature on the growth of C. enzymicum (8156) in tryptone-glucose broth. Data from Table 2.



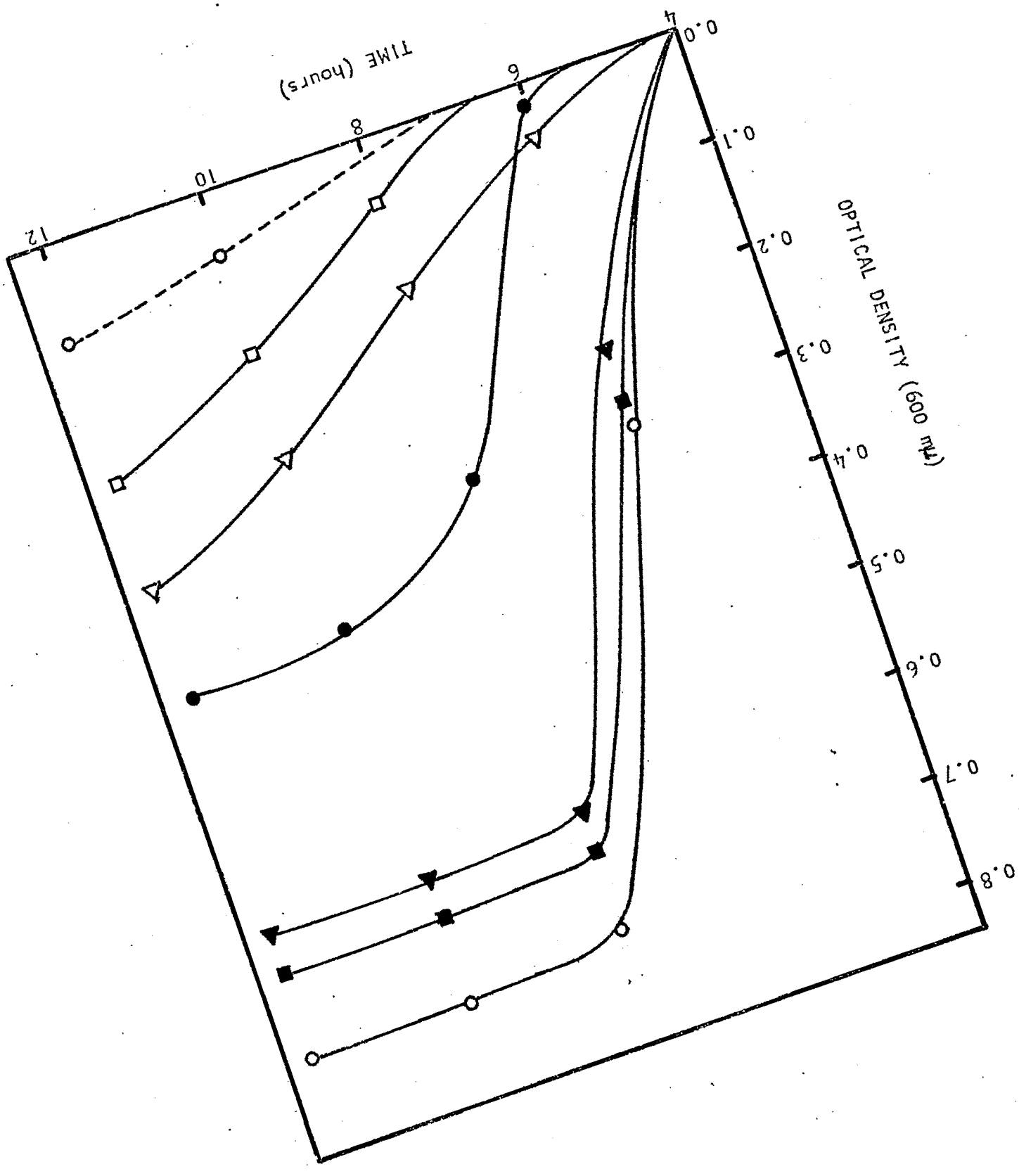
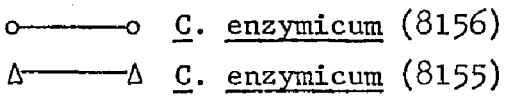

TIME (hours)
4
6
8
10
12
OPTICAL DENSITY (600 mμ)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8

Table 3. The effect of pH on the growth of C. enzymicum (8155 and 8156) in tryptone broth.

Organism	pH 4.0	5.0	6.0	7.0	8.0	9.0	9.5
	Optical density at 600 mμ						
C. enzymicum (8155)	0.00	0.05	0.11	0.145	0.10	0.01	0.00
C. enzymicum (8156)	0.00	0.06	0.125	0.15	0.11	0.025	0.00

Figure 3. The effect of pH on the growth of C. enzymicum in tryptone broth. Data from Table 3.



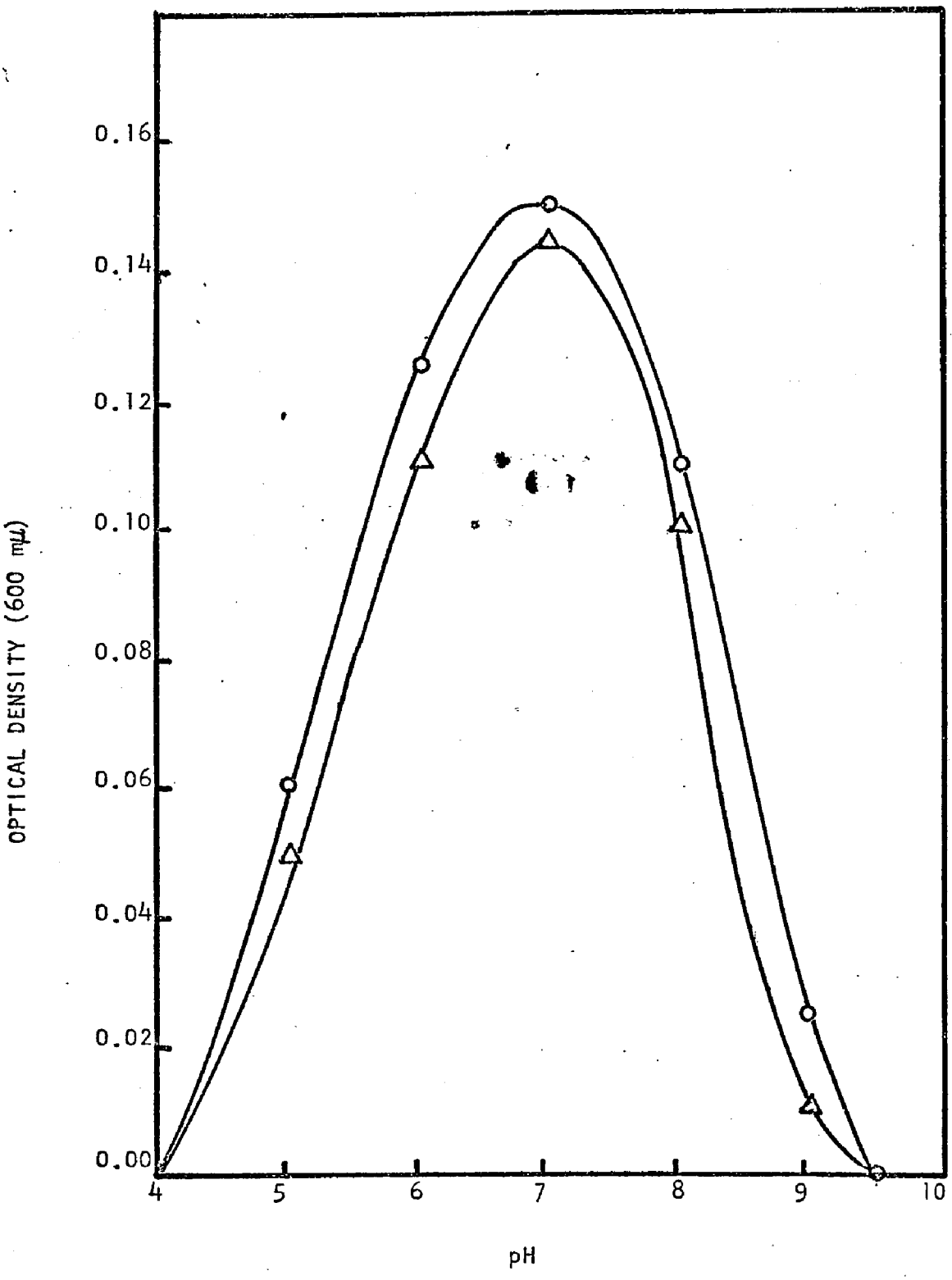
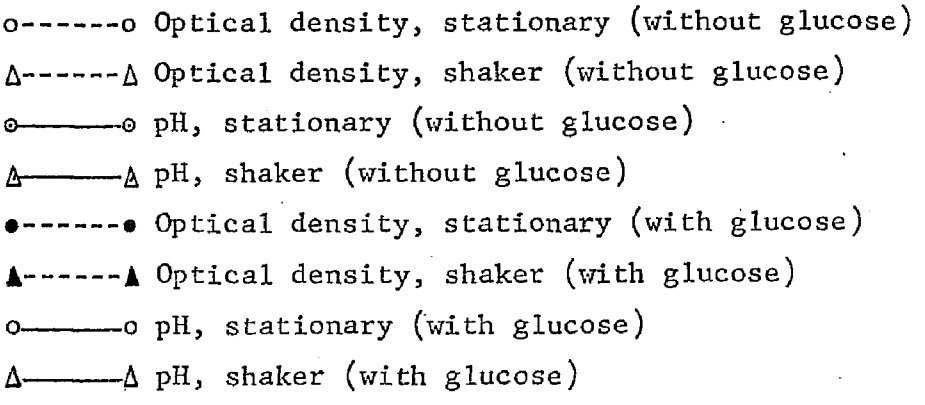

OPTICAL DENSITY (600 mμ)
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
4
5
6
7
8
9
10
pH

Table 4. The effect of aeration on the growth of C. enzymicum (8156)

Time (hrs)	Condition of Incubation: Stationary pH	Stationary O.D. (600 mμ)	Shaker pH	Shaker O.D. (600 mμ)
		Nutrient broth		
0	7.00	0.000	7.00	0.000
5	7.00	0.035	7.00	0.035
7	7.15	0.085	7.20	0.085
9	7.25	0.090	7.30	0.085
11	7.30	0.090	7.30	0.085
24	7.30	0.090	7.30	0.085
		Nutrient broth plus glucose		
0	7.00	0.00	7.00	0.00
5	4.25	0.55	4.25	0.56
7	4.20	0.58	4.20	0.60
9	4.10	0.60	4.10	0.62
11	4.00	0.61	4.00	0.64
24	4.00	0.63	4.00	0.66

Figure 4. The effect of aeration on the growth of C. enzymicum (8156) Data from Table 4.



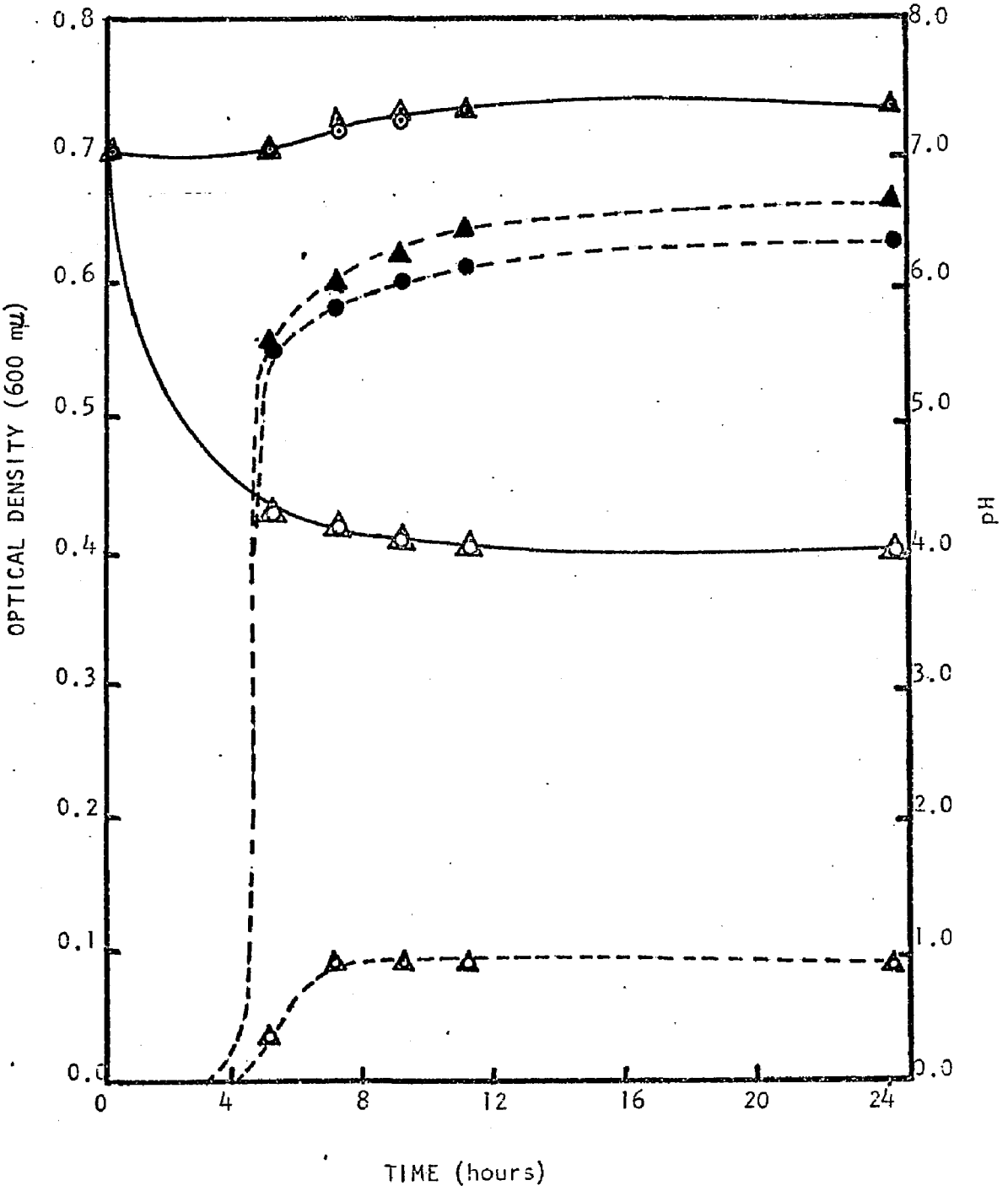

OPTICAL DENSITY (600 mμ)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
pH
0
4
8
12
16
20
24
TIME (hours)

Table 5. Heat resistance of C. enzymicum (8155 and 8156).

C. enzymicum strain	Temperature C	50	55	60	65	70	75
	Time (min)	10	10	3	3	3	3
8155		+	+	-	-	-	-
8156		+	+	-	-	-	-

+, growth; -, no growth.

Table 6. Sodium chloride tolerance of C. enzymicum (8155 and 8156).

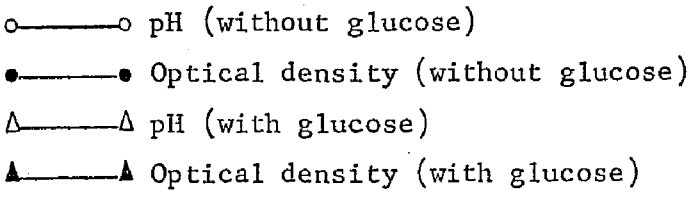
Organism	Percentage Sodium Chloride								
	2.5	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5
C. enzymicum (8155)	+	+	+	+	+	+	+	+	-
C. enzymicum (8156)	+	+	+	+	+	+	+	+	-

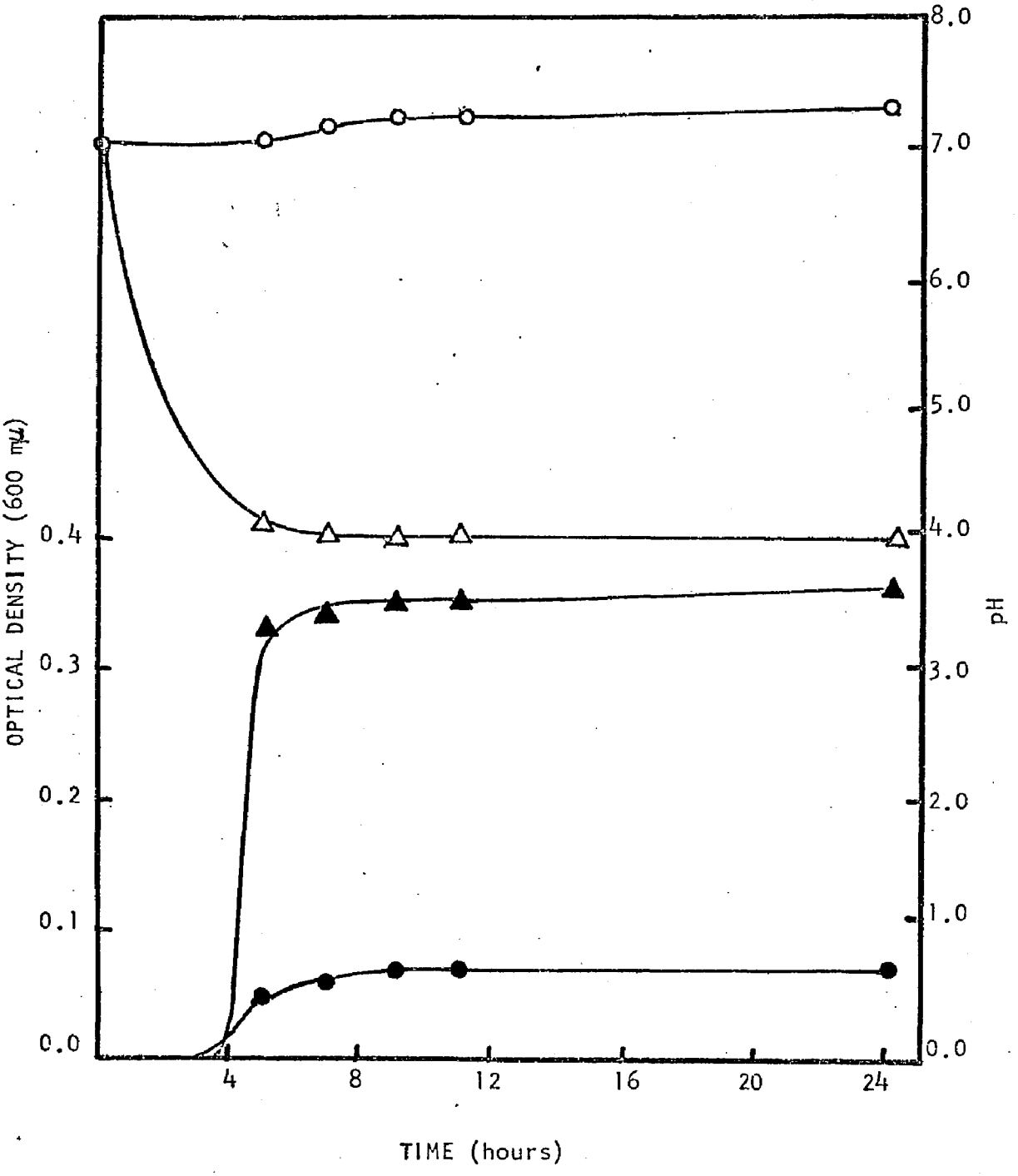
+, growth; -, no growth.

Table 7. Growth of C. enzymicum (8156) in casamino acids medium.

Time (hrs)	Casamino Acids Medium		Casamino Acids Medium Plus Glucose	
	pH	O.D. (600 mμ)	pH	O.D. (600 mμ)
0	7.00	0.00	7.0	0.00
5	7.00	0.05	4.1	0.33
7	7.15	0.06	4.0	0.34
9	7.20	0.07	4.0	0.35
11	7.20	0.07	4.0	0.35
24	7.30	0.07	4.0	0.36

Figure 5. Growth of C. enzymicum (8156) in casamino acids medium. Data from Table 7.




OPTICAL DENSITY (600 mμ)
0.0
0.1
0.2
0.3
0.4
pH
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
4
8
12
16
20
24
TIME (hours)

of this cycle. None of the nitrogenous compounds tested was found to support growth of the organisms.

The effect of thiamin on growth and pigment production. Thiamin has been found to be effective in inducing pigment production and enhancing growth of some corynebacteria, (Schuster, et al., 1959; Pradip, 1966). C. enzymicum did not grow in Braun and Hofmeier's (1927) synthetic medium containing thiamin, and no change in optical density was observed when the organism was grown in tryptone broth supplemented with thiamin.

Oxidative and fermentative utilization of carbohydrates. The organisms were found to utilize a wide range of carbohydrates. All the carbohydrates were utilized fermentatively. Carrier (1963) reported that lactose was not fermented by C. enzymicum. Since these organisms acidified and curdled milk it seemed likely that they possessed the ability to ferment lactose. It was found that C. enzymicum fermented lactose; strain 8156 fermented lactose in 16 hours while the other strain (8155) did so in 36 hours. The organisms also fermented sorbitol and mannitol although Carrier (1963) reported only oxidative attack on mannitol. Strain 8156 fermented mannitol in 48 hours and the other strain did so in 5 days. The organisms were found to ferment ribose and xylose but not arabinose. In all cases strain 8155, the strain studied by Carrier (1963), appeared to be considerably slower in fermentative activity than strain 8156. The results are presented in Table 8.

Various biochemical tests. The results of the various biochemical tests are presented in Table 9. C. enzymicum reduced tellurite and

Table 8. Oxidative and fermentative utilization of carbohydrates by *C. enzymicum*.

O-F Medium Plus Carbohydrate	Utilization by *C. enzymicum* (8155)	Utilization by *C. enzymicum* (8156)
Ribose	F	F
Xylose	F	F
Arabinose	-	-
Rhamnose	-	-
Glucose	F	F
Fructose	F	F
Galactose	F	F
Mannose	F	F
Maltose	F	F
Sucrose	F	F
Lactose	F	F
Trehalose	F	F
Melibiose	F	F
Cellobiose	F	F
Raffinose	-	-
Melizitose	-	-
Starch	F	F
Dextrin	F	F
Inulin	-	-
Glycerol	-	-
Salicin	F	F
Mannitol	F	F
Inositol	-	-
Sorbitol	F	F
Dulcitol	-	-

F, Fermentative utilization;

-, No utilization.

Table 9. Tests for various biochemical properties of C. enzymicum.

Test for	Strain 8155	Strain 8156
Action in litmus milk	A C	A C
Indol production	-	-
Hydrogen sulfide production	-	-
Methyl Red	+	+
Voges-Proskauer reaction	-	-
Growth in KCN	-	-
Nitrate reduction	±	±
Litmus reduction	+	+
Methylene blue reduction	-	-
Tellurite reduction	+	+
Reduction of tetrazolium salts	+	+
Gelatin liquefaction	-	-
Casein hydrolysis	-	-
Sodium hippurate hydrolysis	-	-
Esculin hydrolysis	+	+
Urease	-	-
Amylase	-	-
DNase	-	-
Oxidase	-	-
Cytochrome oxidase	-	-
Catalase	-	-
Peroxidase	-	-

+, Positive;

-, Negative;

A, Acid;

C, Coagulation.

three of the tetrazolium compounds, e.g., nitroblue tetrazolium, iodonitro tetrazolium and tetranitro blue tetrazolium. Nitrite was formed from nitrate only in small amounts. The organisms were found weakly proteolytic, if at all, since they neither liquefied gelatin nor did they digest the casein of milk. They were found equally poor in their hydrolytic activity. However, esculin was hydrolyzed by both strains.

Tests for DNase, oxidase, cytochrome oxidase, catalase and peroxidase. The results of these tests are included in Table 9. The organisms were found to possess none of these enzymes. Whittenbury (1964) reported that L. plantarium was capable of incorporating hematin during growth to produce a typical heme-iron catalase. C. enzymicum, was tested under similar conditions and was found lacking this enzyme. Walker and Kilgour (1965) reported the existence of DPNH-oxidase and DPNH-peroxidase activities in L. casei. C. enzymicum, however, did not show any of these enzyme activities when tested under similar conditions. The cell extract did not appear to be yellow in the case of C. enzymicum. No change in optical density was noticed when the assay system was measured under both aerobic and anaerobic conditions.

Utilization of pentoses. C. enzymicum seemed to grow better in tryptone broth when it was supplemented with ribose or xylose, indicating that these pentoses were utilized by the organisms. Growth and acid production were comparatively more abundant in the medium containing ribose. The growth in the arabinose containing medium was practically the same as that in tryptone broth without any pentose,

indicating that arabinose was not utilized by the organisms (Table 10 and Figure 6).

Growth and acid production were not increased when the organisms were transferred from ribose containing medium to xylose or arabinose containing medium (Table 11), indicating that they did not have the ability for adaptive utilization of xylose and arabinose.

C. enzymicum utilized about two-thirds of the ribose within 36 hours producing only small amount of lactic acid (Table 13 and Figure 10). Carbon dioxide and acetic acid were not detected in ribose breakdown. The ratio of ribose utilized to lactic acid formed was approximately 3.6 to 1, indicating that the organisms did not possess phosphoketolase system, in which case the production of equimolar amounts of acetic acid and lactic acid would have resulted.

When the organisms were grown in the presence of both glucose and ribose, it was noticed that ribose utilization was enhanced in the earlier part of the growth cycle (Table 14 and Figures 11, 12). This probably could be an indication that the organisms obtain their energy from the fermentation of glucose and this energy was mobilized in utilizing more ribose in cellular synthetic mechanisms during the active stage of growth.

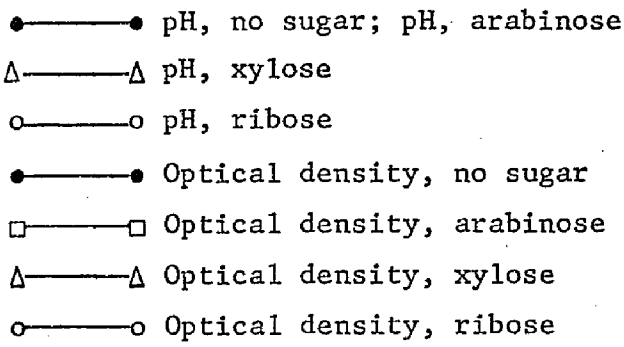
Utilization of glucose. When C. enzymicum was inoculated (5% inoculum) in tryptone broth containing 0.5% glucose, it grew luxuriantly producing considerable acid. It reduced the pH to 4.0, grew to a maximum cell crop and produced considerable amounts of titratable acid and lactic acid within a period of only 12 hours (Table 15 and Figure 13). It did not, however, produce any gas or

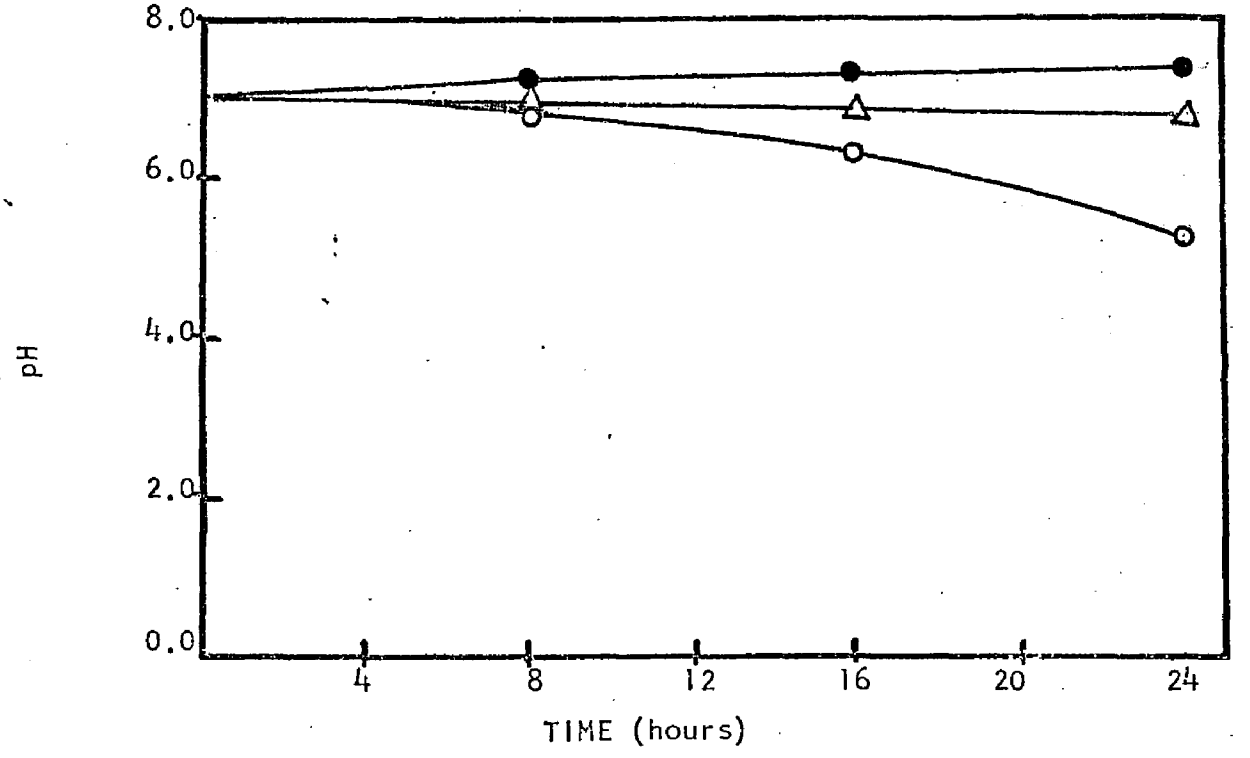
Table 10. Growth and acid production by C. enzymicum (8156) in media containing different pentoses.

Time (hrs)	Tryptone Broth with Added Pentose							
	None		Ribose		Xylose		Arabinose	
	pH	O.D.*	pH	O.D.	pH	O.D.	pH	O.D.
0	7.0	0.00	7.0	0.00	7.0	0.00	7.0	0.00
8	7.2	0.10	6.8	0.14	6.9	0.13	7.2	0.095
16	7.3	0.11	6.0	0.37	6.8	0.20	7.3	0.105
24	7.3	0.11	5.2	0.38	6.7	0.215	7.3	0.11

*Optical density at 600 mμ.

Figure 6. Growth and acid production by C. enzymicum (8156) in media containing different pentoses. Data from Table 10.




8.0
6.0
4.0
2.0
0.0
pH
4
8
12
16
20
24
TIME (hours)

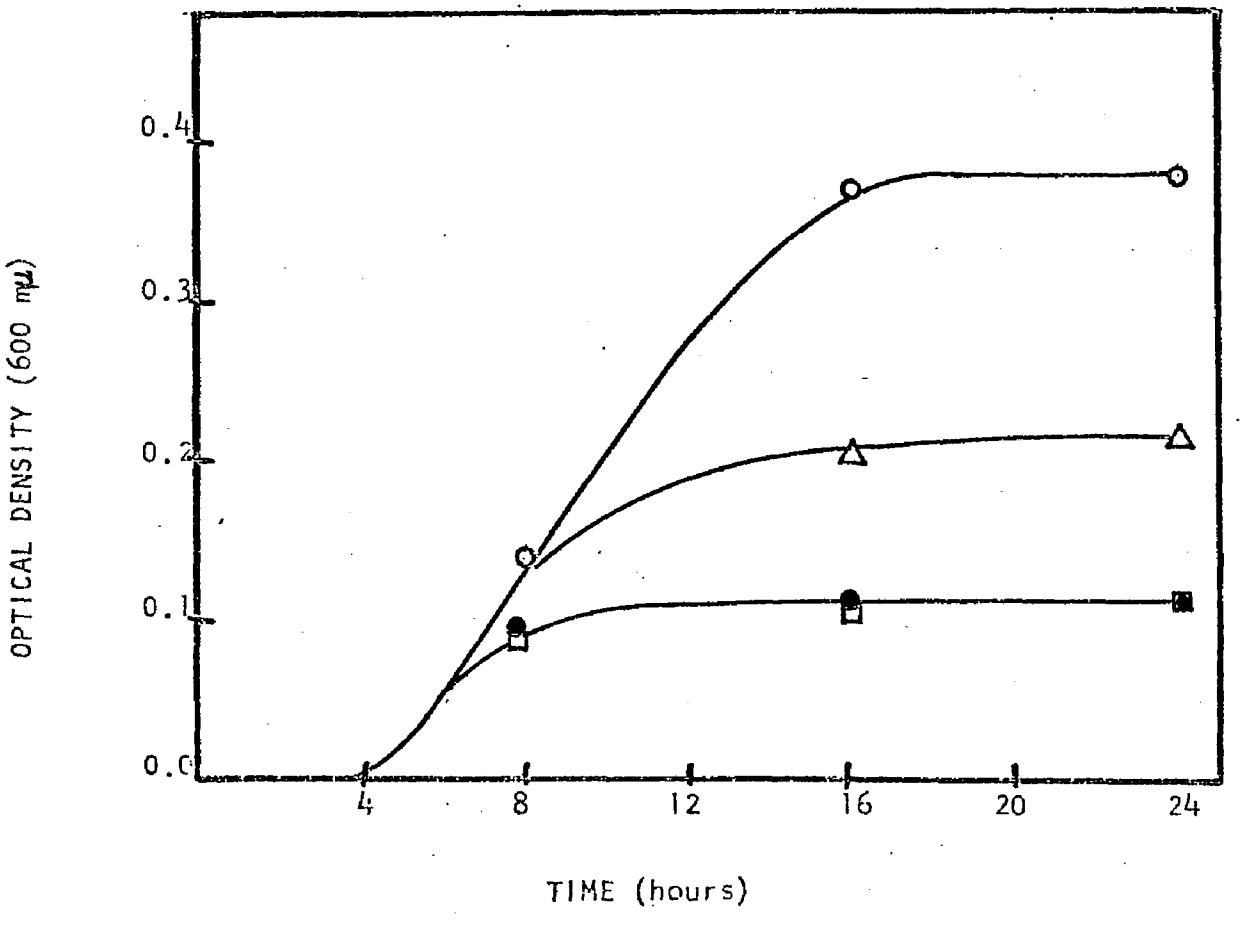

0.4
0.3
0.2
0.1
0.0
OPTICAL DENSITY (600 mμ)
4
8
12
16
20
24
TIME (hours)

Table 11. Test for adaptive utilization of pentoses by C. enzymicum (8156)*.

Cells grown in tryptone broth containing	Cells placed in tryptone broth containing	pH	O.D. (600 mμ)
No sugar	No sugar	7.3	0.11
	Ribose	6.0	0.37
	Xylose	6.8	0.20
	Arabinose	7.3	0.11
Ribose	No sugar	7.3	0.11
	Ribose	5.3	0.36
	Xylose	6.8	0.20
	Arabinose	7.3	0.10
Xylose	No sugar	7.3	0.11
	Ribose	5.1	0.39
	Xylose	6.8	0.19
	Arabinose	7.3	0.10
Arabinose	No sugar	7.3	0.11
	Ribose	5.4	0.27
	Xylose	6.9	0.17
	Arabinose	7.3	0.09

*The results were recorded after 18 hours of incubation at 33 C.

Table 12. Data for the construction of standard curves of ribose, glucose and lactic acid.

Ribose		Glucose		Lactic Acid	
µg/ml	Optical Density at 660 mµ	µg/ml	Optical Density at 500 mµ	µg/ml	Optical Density at 570 mµ
3	0.025	1.5	0.025	1	0.080
5	0.070	3.0	0.060	3	0.275
10	0.155	5.0	0.100	5	0.460
15	0.260	15.0	0.330	10	0.925
20	0.370	30.0	0.660		
30	0.550				

Figure 7. Standard curve of ribose.
Data from Table 12.

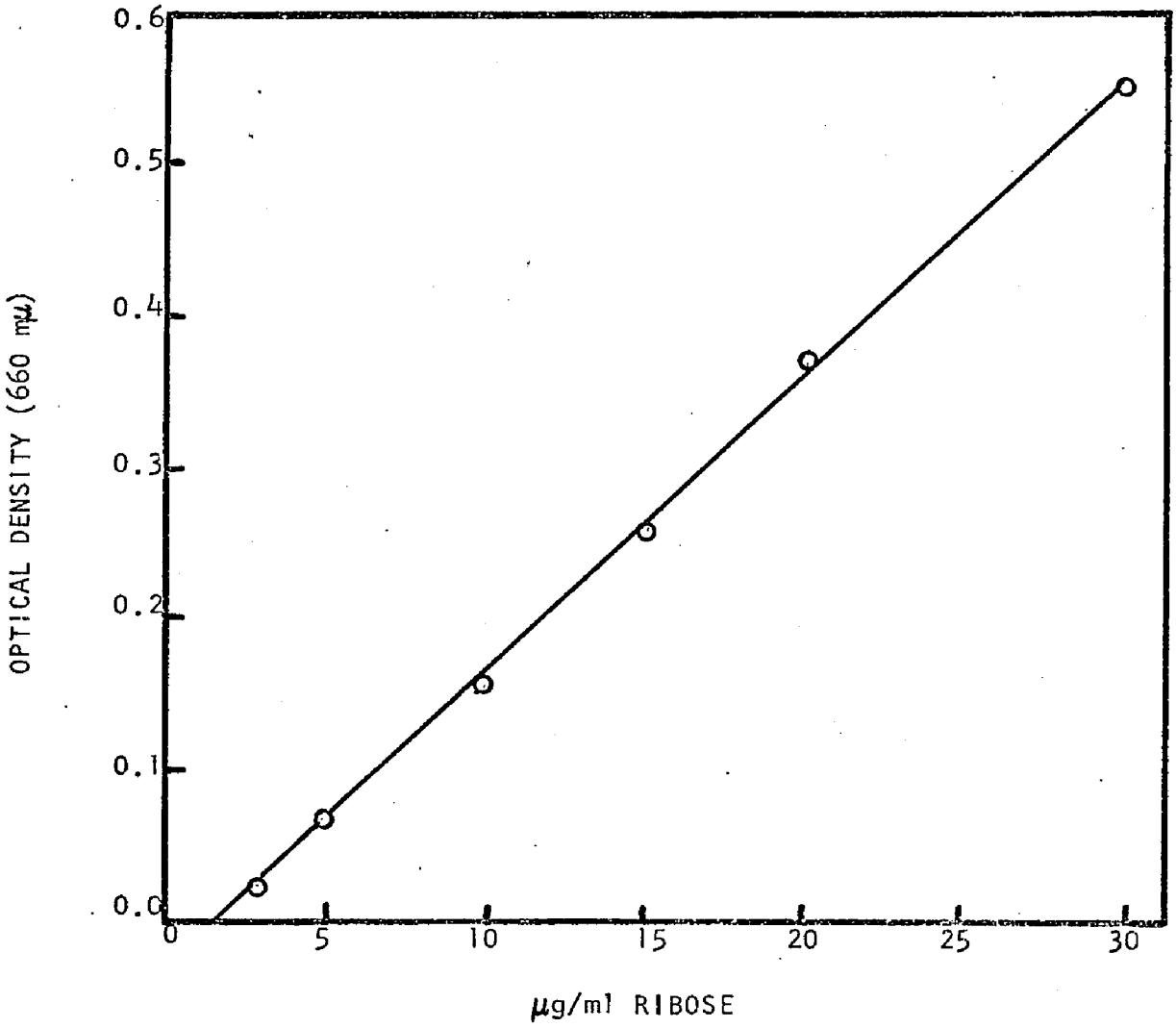

OPTICAL DENSITY (660 mμ)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
20
25
30
μg/ml RIBOSE

Figure 8. Standard curve of glucose.
Data from Table 12.

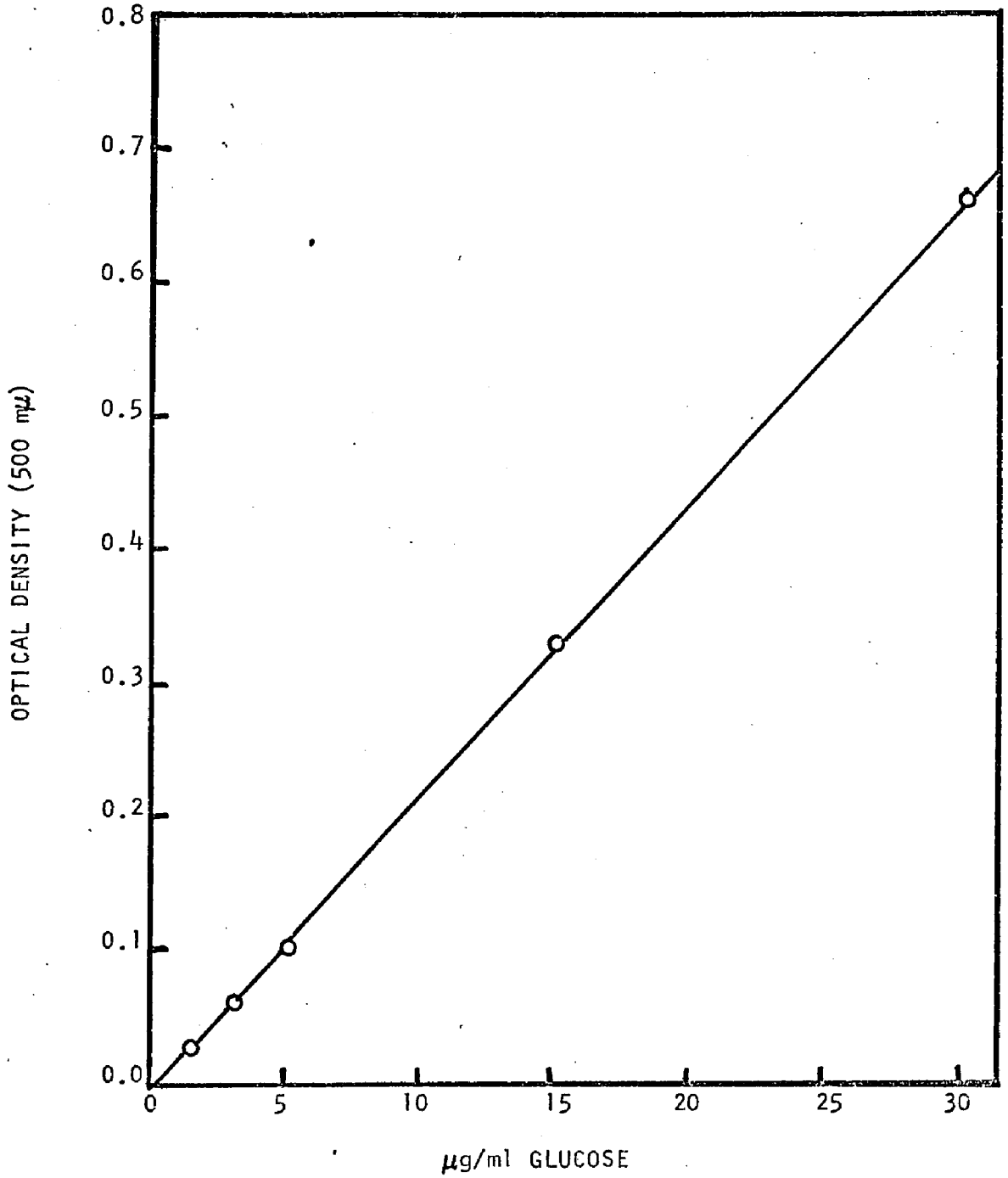

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OPTICAL DENSITY (500 mμ)
0
5
10
15
20
25
30
μg/ml GLUCOSE

Figure 9. Standard curve of lactic acid.
Data from Table 12.

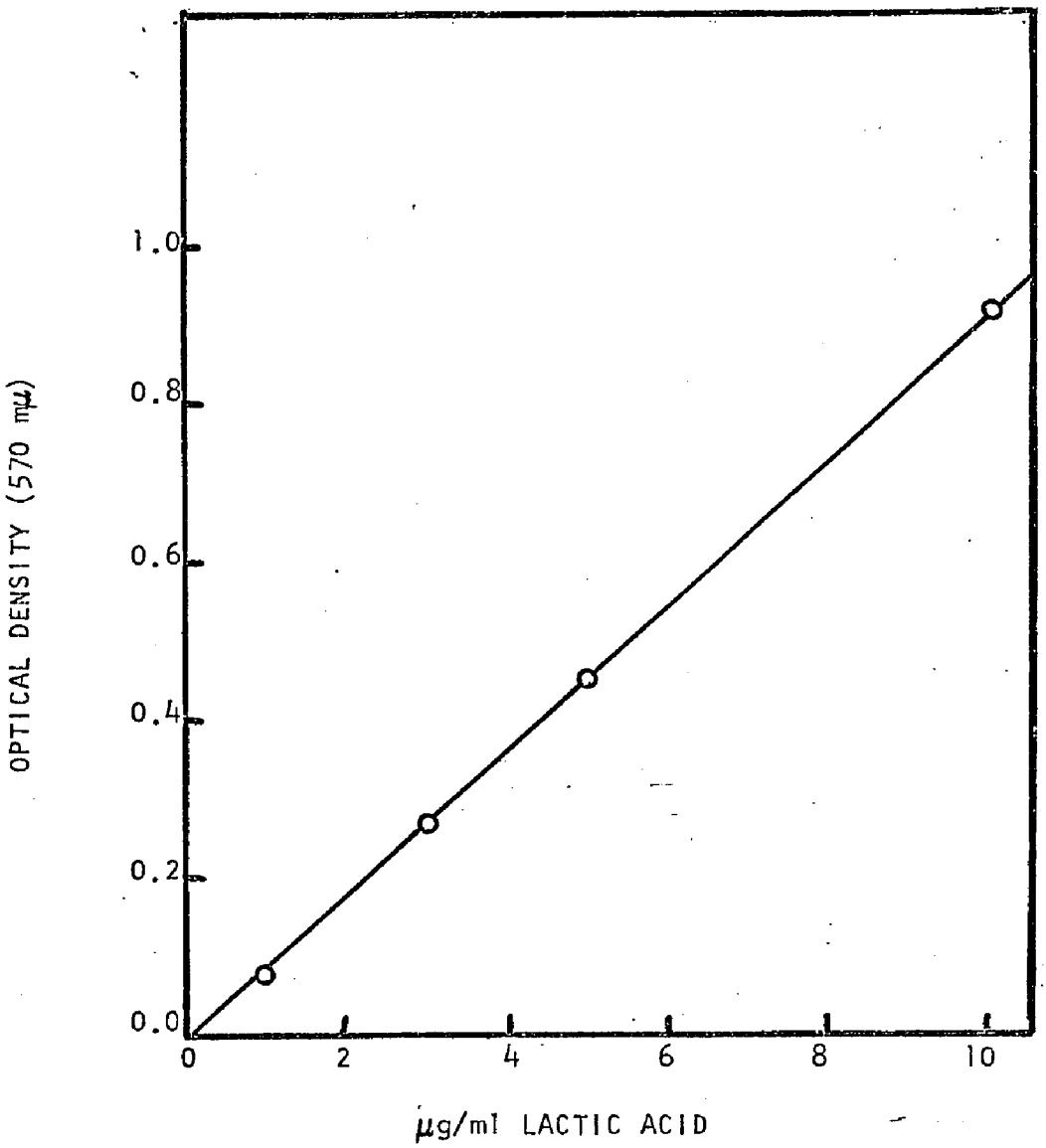
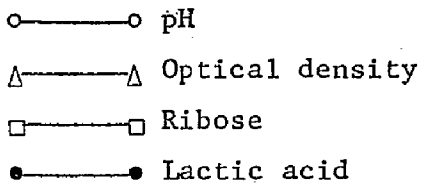

OPTICAL DENSITY (570 mμ)
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
μg/ml LACTIC ACID

Table 13. Utilization of ribose by C. enzymicum (8156).

Tryptone broth plus	Time (hrs)	Optical Density at 600 mμ	pH	Ribose μg/ml	Lactic Acid μg/ml
No ribose	0	0.00	7.0	20	73
	12	0.11	7.1	0	73
	24	0.13	7.2	0	73
	36	0.14	7.25	0	73
Ribose	0	0.00	7.0	2200	73
	12	0.21	6.2	2000	220
	24	0.39	5.2	800	318
	36	0.40	5.0	740	395

Figure 10. Utilization of ribose by C. enzymicum (8156). Data from Table 13.



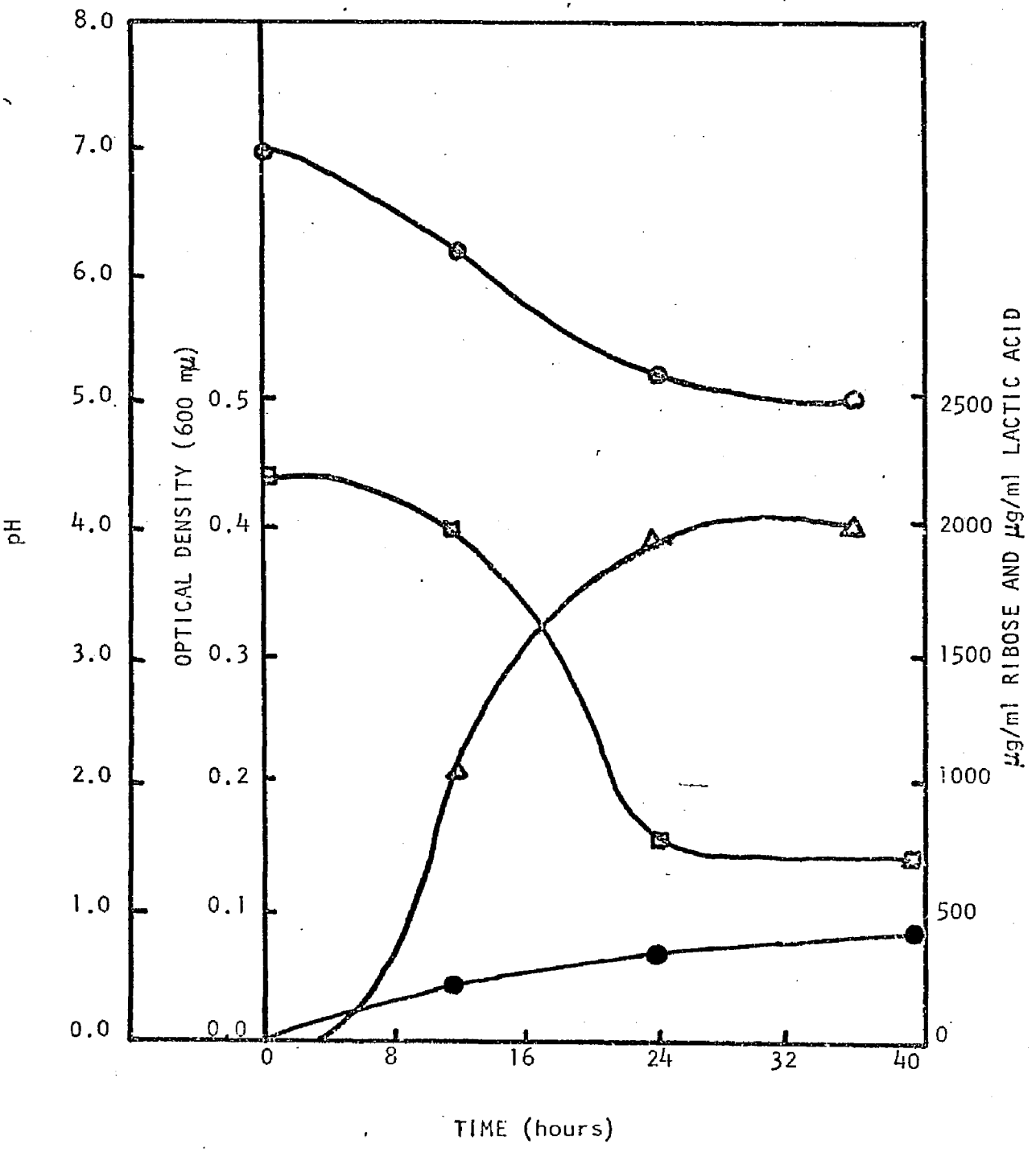
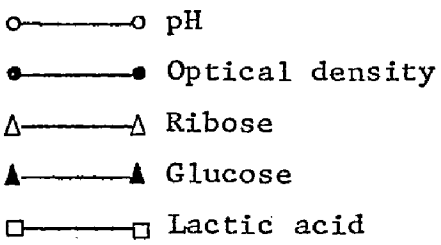

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
pH
0.5
0.4
0.3
0.2
0.1
0.0
OPTICAL DENSITY (600 mμ)
2500
2000
1500
1000
500
0
μg/ml RIBOSE AND μg/ml LACTIC ACID
0
8
16
24
32
40
TIME (hours)

Table 14. Utilization of ribose by C. enzymicum (8156) in the presence of glucose.

Time (hrs)	O.D. (600 mμ)	pH	Ribose (μg/ml)	Glucose (μg/ml)	Lactic Acid (μg/ml)
0	0.00	7.0	2200	750	73
12	0.51	5.2	1400	0	640
24	0.55	4.6	1070	0	680
36	0.60	4.4	840	0	720
0	0.00	7.0	2200	1650	73
12	0.68	4.1	1400	0	1480
24	0.75	4.0	1120	0	1520
36	0.84	4.0	838	0	1580

Figure 11. Utilization of ribose by C. enzymicum (8156) in the presence of 0.07% glucose. Data from Table 14.



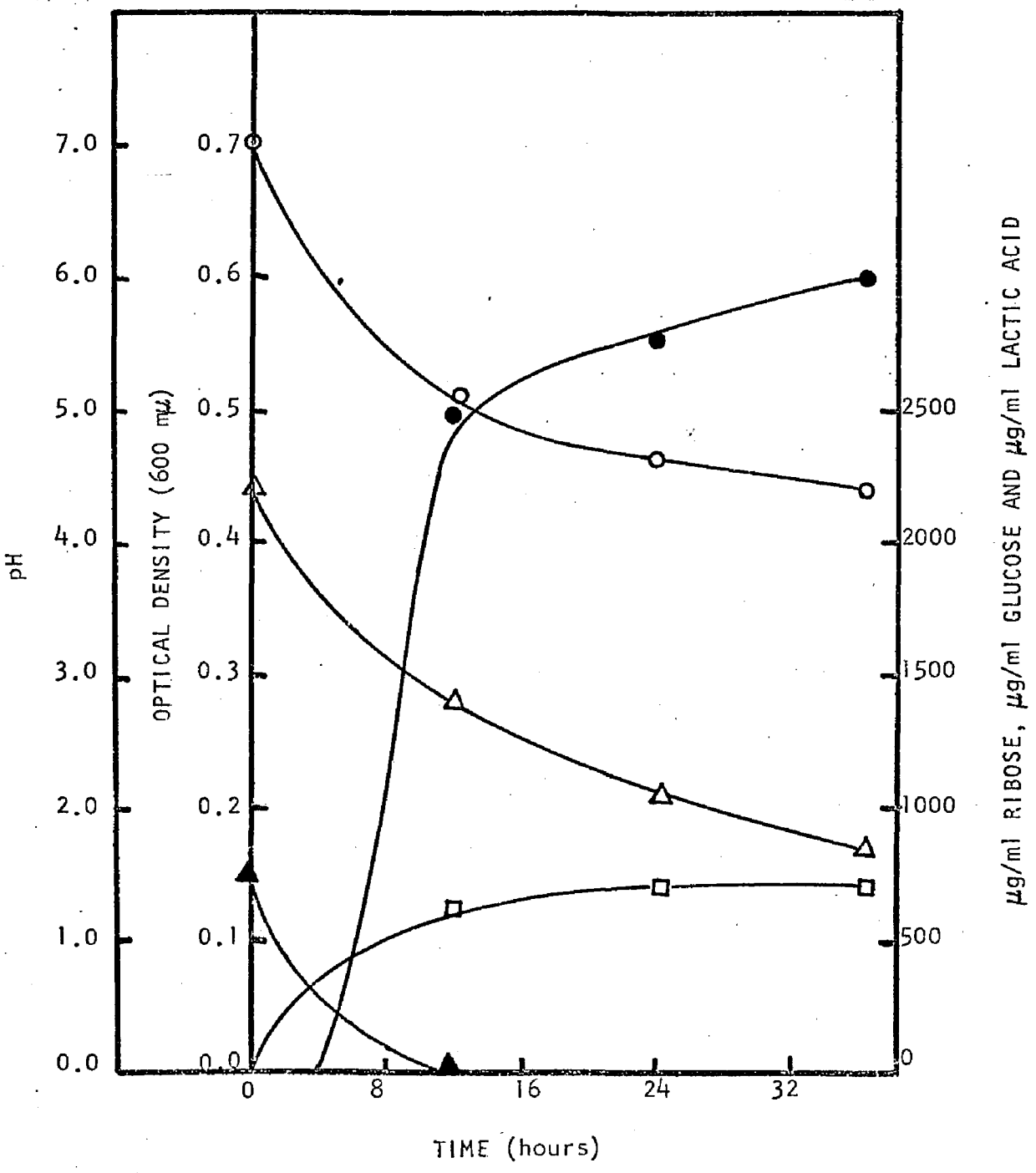
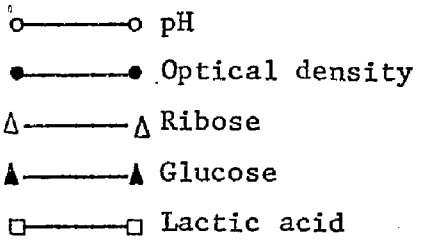

pH
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
OPTICAL DENSITY (600 mμ)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
2500
2000
1500
1000
500
0
μg/ml RIBOSE, μg/ml GLUCOSE AND μg/ml LACTIC ACID
0
8
16
24
32
TIME (hours)

Figure 12. Utilization of ribose by C. enzymicum (8156) in the presence of 0.16% glucose. Data from Table 14.



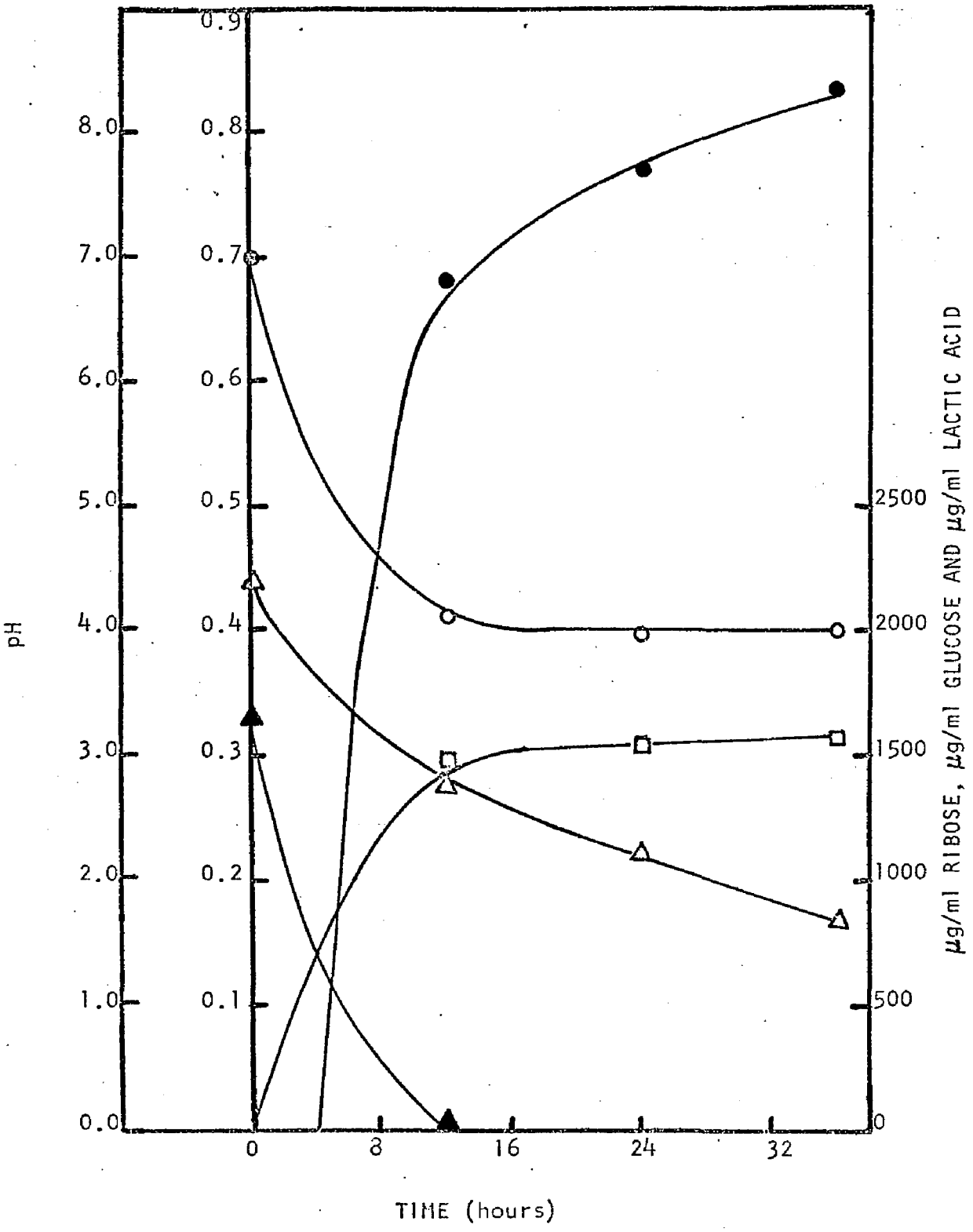
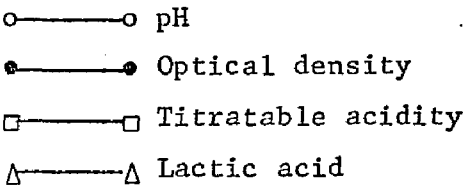
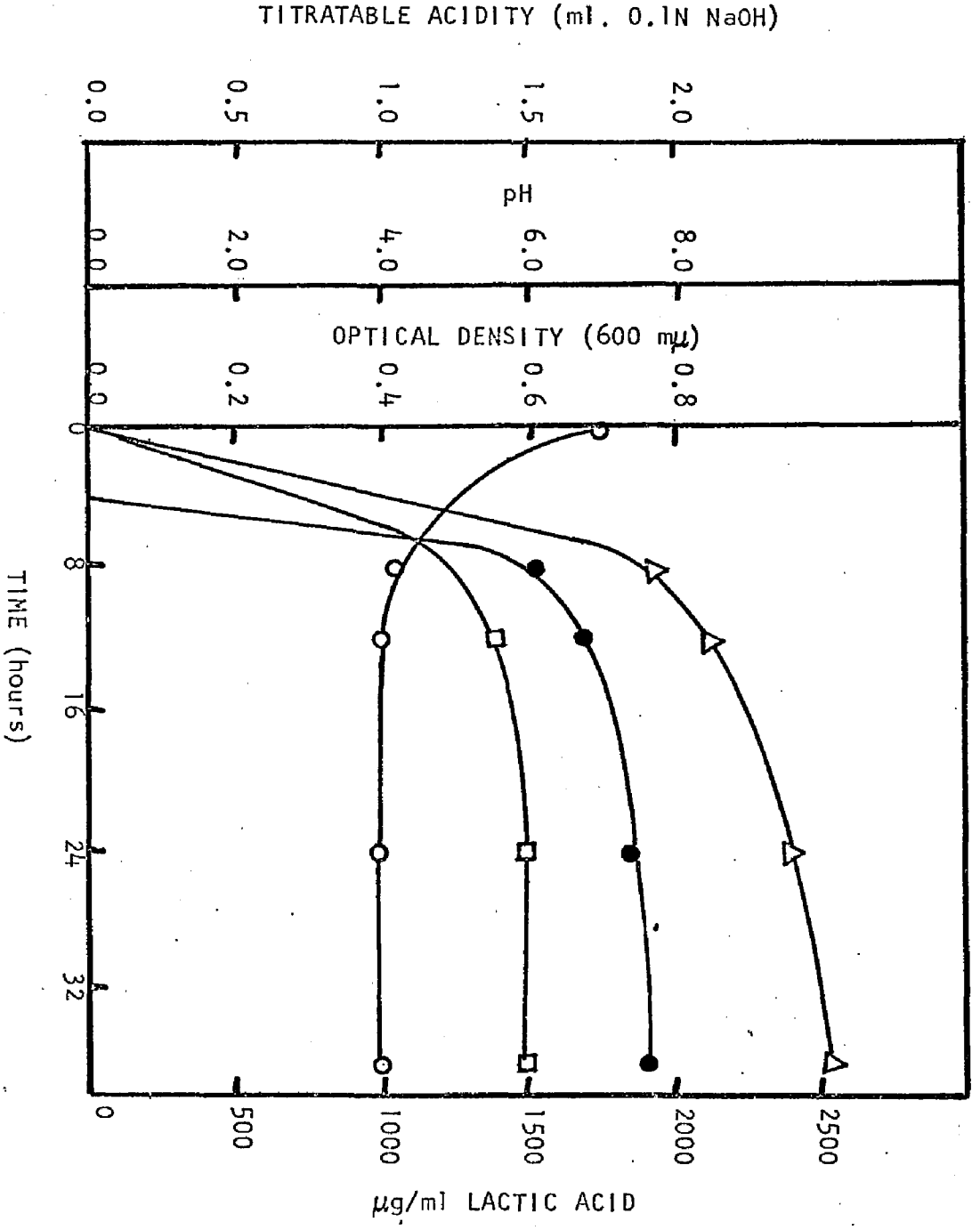

pH
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
2500
2000
1500
1000
500
0
µg/ml RIBOSE, µg/ml GLUCOSE AND µg/ml LACTIC ACID
0
8
16
24
32
TIME (hours)

Table 15. The effect of glucose on growth and acid production by C. enzymicum (8156).

Time (hrs)	Optical Density (at 600 mμ)	pH	Titratable Acidity	Volatile Acidity	Lactic Acid μg/ml
0	0.00	7.0	0.250	none	230
8	0.61	4.2	1.200	"	1950
12	0.67	4.0	1.395	"	2125
24	0.74	4.0	1.500	"	2400
36	0.77	4.0	1.500	"	2550

Figure 13. The effect of glucose on growth and acid production by C. enzymicum (8156). Data from Table 15.




TITRATABLE ACIDITY (ml. 0.1N NaOH)
0.0
0.5
1.0
1.5
2.0
pH
0.0
2.0
4.0
6.0
8.0
OPTICAL DENSITY (600 mμ)
0.0
0.2
0.4
0.6
0.8
TIME (hours)
0
8
16
24
32
0
500
1000
1500
2000
2500
μg/ml LACTIC ACID

volatile acids from glucose breakdown. C. enzymicum appeared to be a strongly fermentative organism.

Since most corynebacteria are highly oxidative, the type species along with two other less fermentative species, C. kutscheri and C. striatum, were compared with C. enzymicum. It was noticed that the other species behaved quite differently from C. enzymicum as regards fermentative activity. They produced only very small amount of lactic acid; growth and titratable acidity were also considerably less (Table 16 and Figures 14, 15).

Since C. enzymicum produced a considerable amount of lactic acid from glucose, a glucose dissimilation study was carried out to quantitate the amount of glucose utilized to that of lactic acid formed. It was revealed by this study that lactic acid was the only detectable fermentation product and the yield of lactic acid turned out to be nearly 100% of theoretical, (Tables 17, 18 and Figure 16), indicating that C. enzymicum is homofermentative.

The optical activity of lactic acid. Examination of the optical type of lactic acid produced by C. enzymicum revealed that it produced almost entirely L (+) lactic acid (Table 20).

Pathogenicity

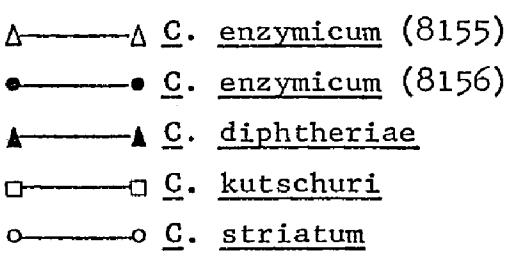
Mellon (1917) reported that C. enzymicum is pathogenic to rabbits, guinea pigs and mice. The Schwarz strain (ATCC 8156) was found to be non-pathogenic for rabbits.

Table 16. Comparative study of fermentative activity of some species of the genus Corynebacterium in tryptone-glucose broth.

Time (hrs)		Organism				
		C. enzymicum (8155)	C. enzymicum (8156)	C. diphtheriae (mitis)	C. kutscheri	C. striatum
0	O.D. (600 mμ)	0.000	0.000	0.000	0.000	0.000
	pH	7.00	7.00	7.00	7.00	7.00
	Titratable acid*	0.250	0.250	0.250	0.250	0.250
	Lactic acid**	230	230	230	230	230
12	O.D. (600 mμ)	0.740	0.670	0.030	0.200	0.125
	pH	4.00	4.00	6.8	6.3	6.6
	Titratable acid	1.395	1.395	0.145	0.345	0.145
	Lactic acid	2400	2200	22	46	29
24	O.D. (600 mμ)	0.800	0.740	0.070	0.330	0.320
	pH	4.00	4.00	6.60	5.40	6.10
	Titratable acid	1.500	1.500	0.145	0.345	0.245
	Lactic acid	2500	2400	60	160	140
36	O.D. (600 mμ)	0.825	0.775	0.090	0.350	0.350
	pH	4.00	4.00	6.10	5.20	5.30
	Titratable acid	1.500	1.500	0.345	0.545	0.545
	Lactic acid	2600	2500	90	200	200
48	O.D. (600 mμ)	0.850	0.810	0.100	0.375	0.360
	pH	4.00	4.00	6.00	5.15	5.20
	Titratable acid	1.500	1.500	0.345	0.545	0.545
	Lactic acid	2800	2750	120	250	240

*ml 0.1N NaOH;
**μg/ml

Figure 14. Titratable acidity and pH of tryptone-glucose broth cultures of some species of the genus Corynebacterium. Data from Table 16.



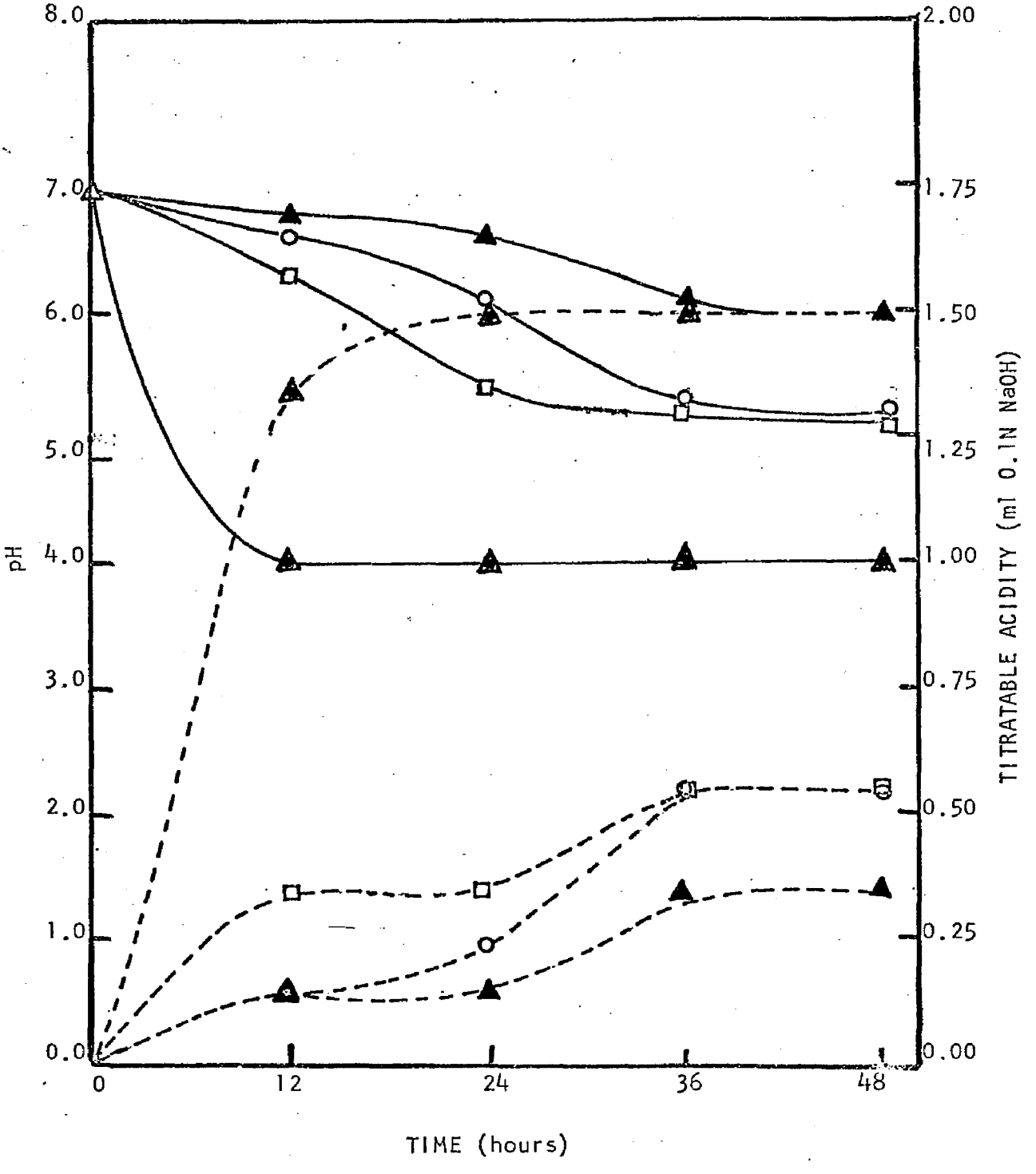
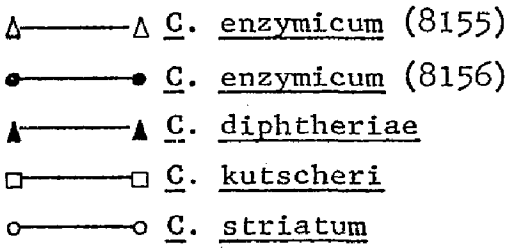

8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
pH
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
TITRATABLE ACIDITY (ml 0.1N NaOH)
0
12
24
36
48
TIME (hours)

Figure 15. Growth and lactic acid production by some species of the genus Corynebacterium. Data from Table 16.



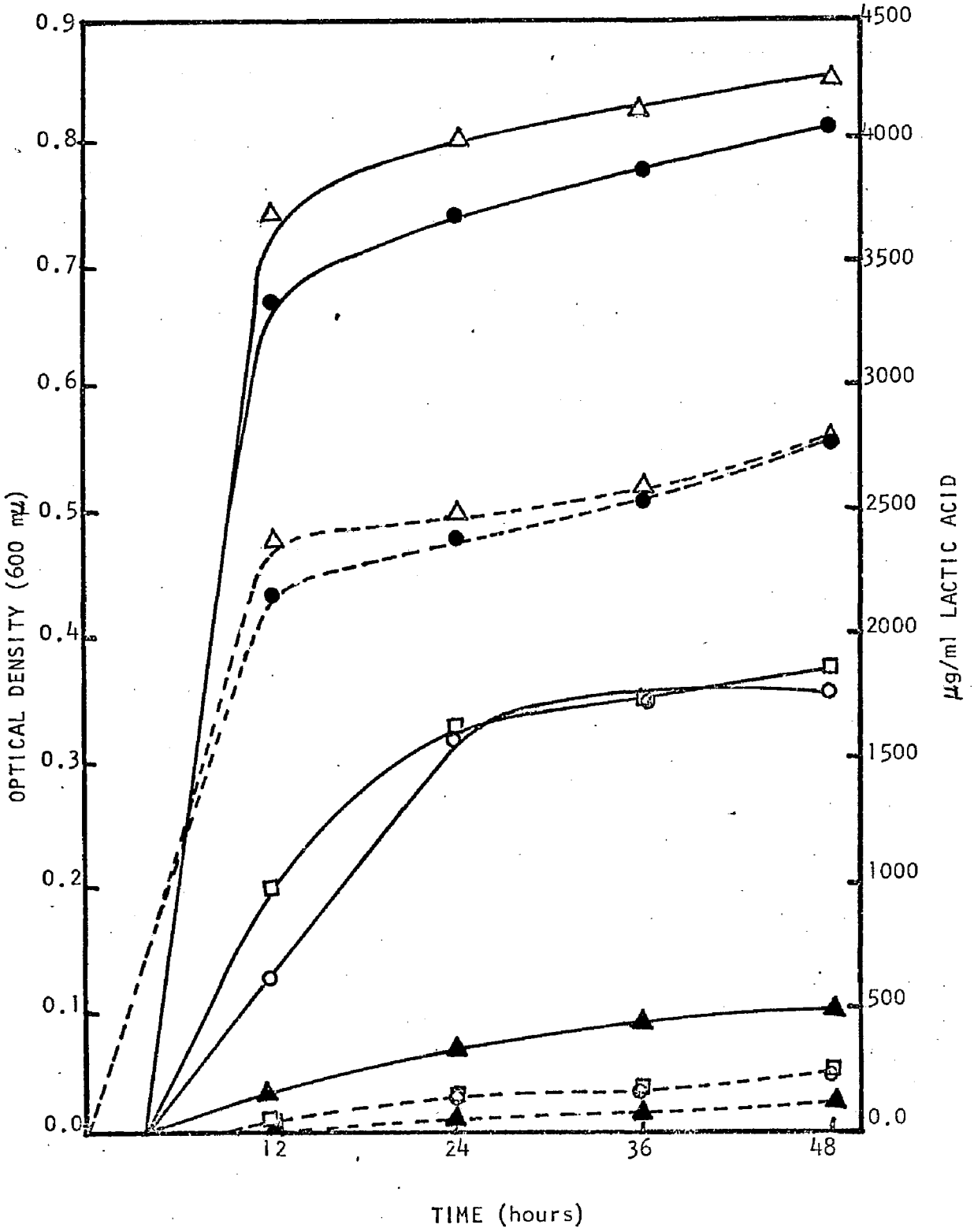
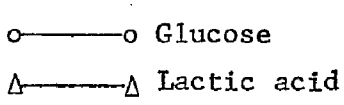

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OPTICAL DENSITY (600 mμ)
4500
4000
3500
3000
2500
2000
1500
1000
500
0.0
μg/ml LACTIC ACID
12
24
36
48
TIME (hours)

Table 17. Glucose dissimilation by C. enzymicum (8156).

Time (hrs)	Glucose Present µg/ml*	Lactic Acid Present µg/ml*
0	2450	0
8	0	2095
16	0	2200
24	0	2335
36	0	2385

*Corrected for initial content of medium (250 µg/ml of glucose and 140 µg/ml of lactic acid were initially present in the medium).

Figure 16. Glucose dissimilation by C. enzymicum (8156)
Data from Table 17.



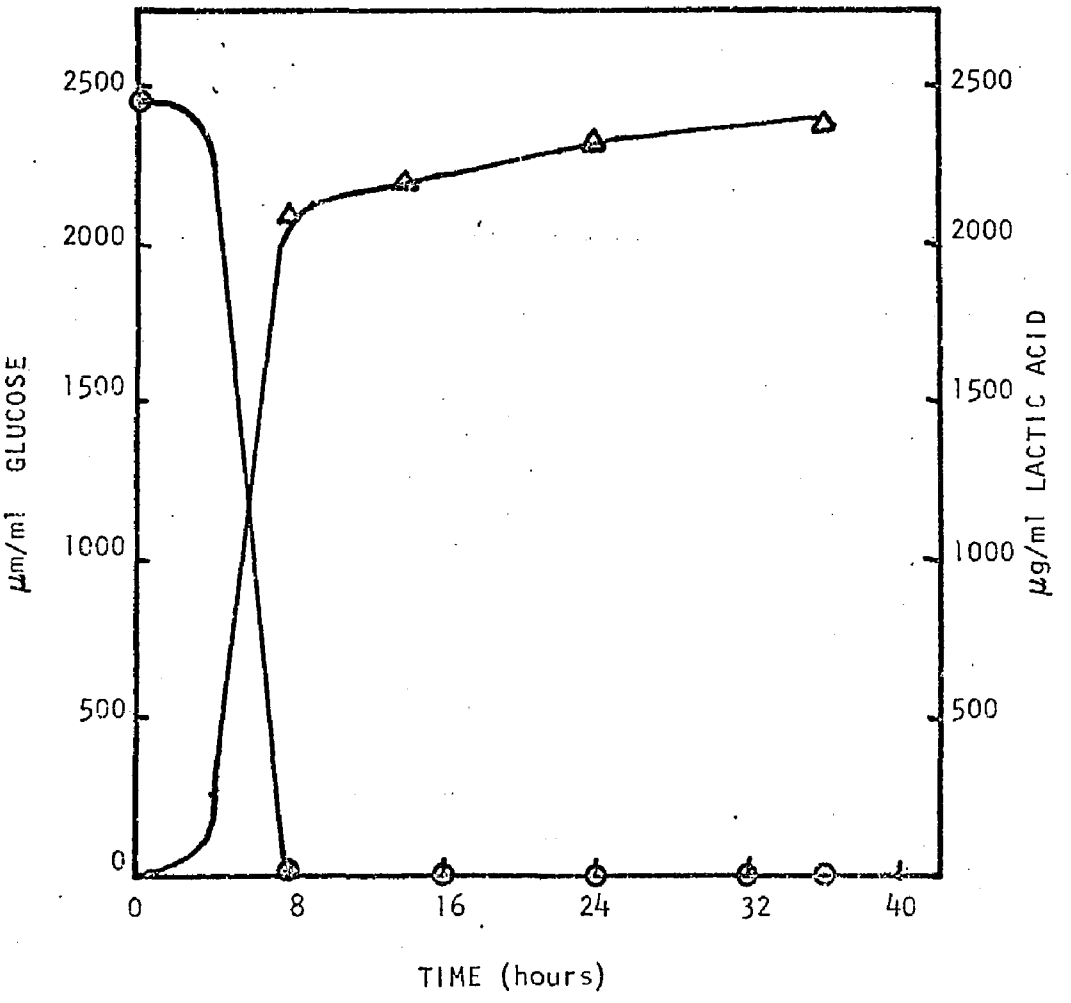

2500
2000
1500
1000
500
0
µm/ml GLUCOSE
µg/ml LACTIC ACID
0
8
16
24
32
40
TIME (hours)

Table 18. Ratio of lactic acid produced to glucose utilized by C. enzymicum (8156).

Time (hrs)	Glucose Utilized µg/ml*	Lactic Acid Produced µg/ml*	Ratio Lactic Acid Formed / Glucose Used
0	0	0	0.00
8	2450	2095	0.86
16	2450	2200	0.90
24	2450	2335	0.95
36	2450	2385	0.97

*Values of glucose and lactic acid were corrected for those present initially in the medium.

Table 19. Data for the construction of standard curve of L(+) lactic acid.

μ mole of L(+) Lactic acid*	μg/0.1 ml of L(+) lactic acid*	Optical Density at 366 mμ
0.1	9	0.250
0.2	18	0.335
0.4	36	0.460
0.5	45	0.510
0.8	72	0.710
1.0	90	0.820

*Sodium salt

Figure 17. Standard curve for L (+) lactic acid.
Data from Table 19.

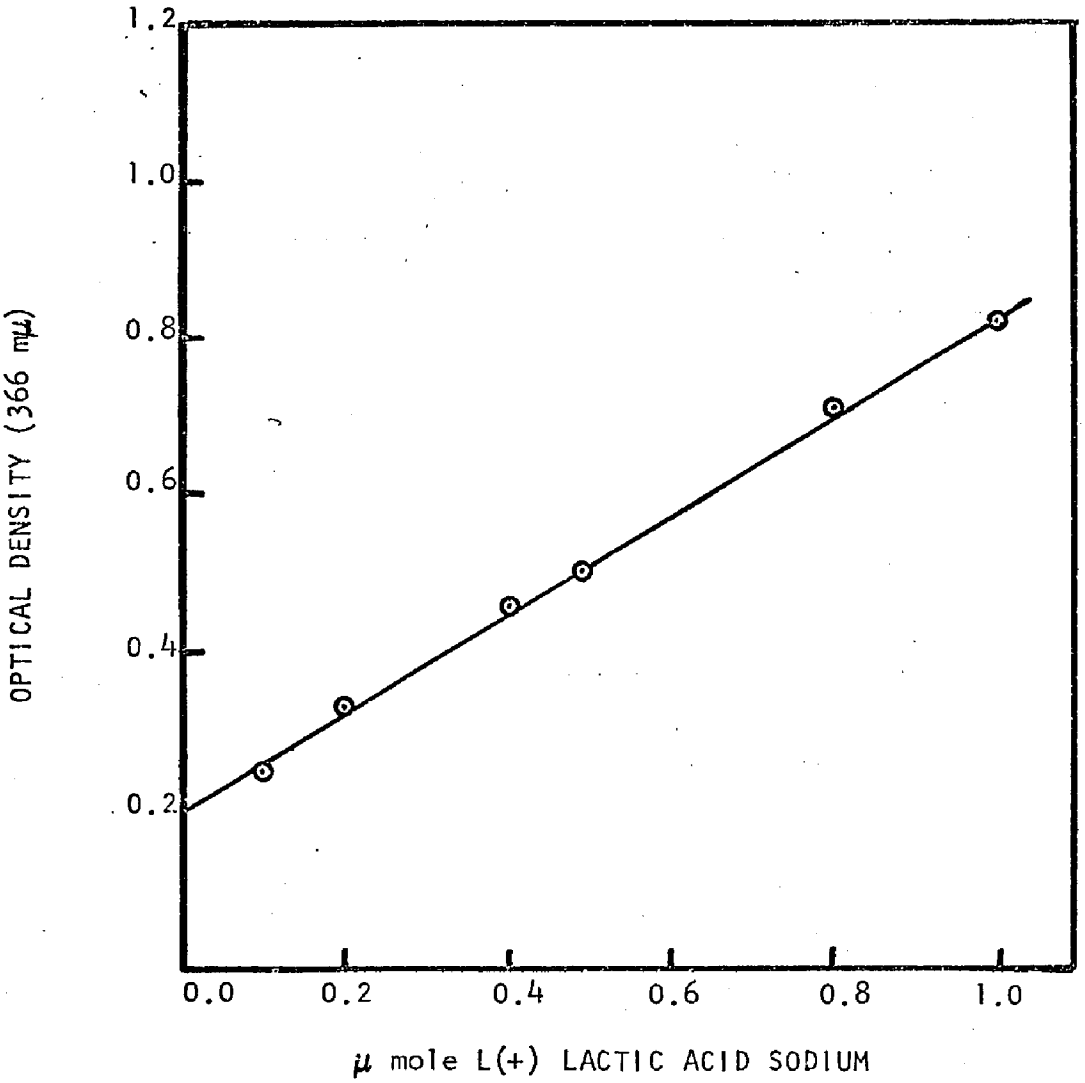

1.2
1.0
0.8
0.6
0.4
0.2
OPTICAL DENSITY (366 mμ)
0.0
0.2
0.4
0.6
0.8
1.0
μ mole L(+) LACTIC ACID SODIUM

Table 20. Optical activity of lactic acid produced by C. enzymicum (8156).

Total lactic acid mg/ml	L (+) lactic acid mg/ml	Ratio $\frac{\text{L (+) lactic acid}}{\text{Total lactic acid}}$
1.984	1.962	0.988

Characterization of DNA by base composition

Marmur's method (1961) was found suitable for the isolation of DNA but the BPES buffer of Massie and Zimm (1965) was more satisfactory to effect subsequent lysis of cells. It was observed that when the cell suspension was heated to 70 C for 20 minutes prior to lysis (as suggested by Massie and Zimm, 1965 to inactivate the nucleases) the yield of DNA was increased. It was found that C. enzymicum cells could not be lysed when treated with lysozyme alone or even when the lysozyme treatment was followed by the addition of 25% sodium lauryl sulfate and heated to 60 C for 10 minutes. Lysis readily occurred, however, when the cell suspension was first heat treated and then treated with lysozyme followed by the addition of sodium lauryl sulfate at either 37 C or at room temperature (25 C). The yield of DNA by this method was approximately 2 mg (dry wt) per liter of culture.

Vischer and Chargaff (1948) recommended 98% formic acid for the hydrolysis of DNA. Wyatt (1951) reported that hydrolysis with 98% formic acid at 175 C for 30 minutes liberated all the bases from nucleic acids. In this study it was found that hydrolysis at 175 C for 60 minutes yielded only partial hydrolysis, liberating only adenine and guanine. Complete hydrolysis of DNA was effected at 185 C for 90 minutes.

Whatman No. 1 filter paper was suitable for paper chromatography as recommended by Kream and Chargaff (1952). The bases were nicely separated by using isopropanol-HCl solvent system. According to Bendich (1957 b), this solvent system, in addition to its high

resolving power, has an added advantage over the neutral or ammoniacal systems, since it permits the analysis of larger quantities of guanine. Bendich, using descending chromatography with the isopropanol-HCl solvent system on Whatman No. 1 filter paper, found the following Rf values: 0.25 for guanine, 0.36 for adenine, 0.47 for cytosine and 0.77 for thymine. In this study using the same procedure comparable Rf values were obtained, namely 0.26 for guanine, 0.37 for adenine, 0.47 for cytosine and 0.76 for thymine. Twenty-eight hours were required for the solvent front to run 40 cm.

The differential extinction technique of Bendich (1957 b) was chosen as a quantitative method for calculation of the bases. Since 0.1N HCl extracts of filter paper show a low but variable absorption in the ultraviolet, the differential extinction technique of Bendich was found suitable to overcome this difficulty. According to Bendich (1957 b), this technique gives very accurate results.

The results of this study are presented in Tables 21 and 22. The G-C content is expressed as mole percent G+C (G+C/A+T + G+C) (Table 22) in order to compare this value with the mole per cent G+C values obtained for other microorganisms reported by Marmur et al. (1963). The mole per cent G+C of C. enzymicum (8156) was found to be 39 in this study (Table 22). The relative percent of adenine and thymine was approximately same; the percent of guanine was slightly higher than the percent of cytosine. The amount of purine was more than pyrimidine in the DNA hydrolysate (Table 21).

Table 21. The base composition of C. enzymicum (8156) DNA calculated by the differential extinction technique of Bendich (1957 b)*.

Trial No.	Relative Percent				Ratio		
	Guanine	Adenine	Cytosine	Thymine	A/T	G/C	Purine/Pyrimidine
1	21.3	30.7	18.2	29.8	1.03	1.17	1.08
2	21.2	31.1	18.0	30.6	1.02	1.18	1.08
3	20.5	31.0	17.8	30.9	1.00	1.15	1.06
4	20.6	31.5	17.5	29.4	1.07	1.18	1.11
Average	20.9	31.1	17.9	30.2	1.03	1.17	1.09

*The DNA was hydrolyzed by 98% formic acid followed by descending chromatography on Whatman No. 1 filter paper using isopropanol-HCl solvent system.

Table 22. The relative concentration of bases in the DNA of C. enzymicum (8156). (method of Bendich, 1957 b).

Trial No.	A + T	G + C	$\frac{A + T}{G + C}$	$\frac{G + C}{A + T}$	Mole % G + C*
1	60.5	39.5	1.53	0.653	39.50
2	61.7	39.2	1.57	0.635	38.85
3	61.9	38.3	1.62	0.619	38.22
4	60.9	38.1	1.60	0.626	38.48
Average	61.3	38.8	1.59	0.633	38.76

* $\frac{G + C}{A + T + G + C}$

DISCUSSION

In his critique on bacterial taxonomy, Leifson (1966) has stated, "unfortunately, many bacteriologists tend to regard a culture of bacteria as if it were a chemical and to accept the label at its face value much as they would the label on a bottle from a reputable manufacturer". He also pointed out that many of the cultures of the American Type Culture Collection have not been properly identified or do not conform to the descriptions in the literature or as recorded in Bergey's Manual. Many of the original isolates, as is the case with C. enzymicum, are no longer available and the new strains need proper identification. The "conventional taxonomy" (Leifson, 1966) apparently has many shortcomings. With the recent advances in molecular biology additional criteria have become available for the differentiation of species. One of these is the base composition of the DNA of organisms. The purpose of the present investigation was to apply both conventional and new techniques to a taxonomic study of the cultures designated C. enzymicum (Mellon) Eberson.

The results of this study indicate that the two strains isolated by Schwarz (1941) present a close resemblance to the original isolates of Mellon (1917). Like Mellon's isolates, they are gram-positive, non-motile, non-spore forming, pleomorphic rods showing coccoid forms. They also stain well with methylene blue. The colonies on agar are very small. Glucose is also observed to enhance growth of these organisms. The thermal death time of these strains is 60 C for 3 minutes, very close to that reported by Mellon for his isolates. They

ferment glucose, lactose, maltose and dextrin without gas production as observed by Mellon. They are also found to acidify and coagulate milk, and to reduce a small amount of nitrate to nitrite. None of the strains liquefy gelatin. However, unlike Mellon's observation, these strains are not found to show the dimorphic feature (rods to cocci to rods). Their optimum temperature for growth is between 30 and 33 C instead of 37 C. Mellon (1917) reported that his isolates were pathogenic to rabbits, guinea pigs and mice, but the Schwarz strain (ATCC 8156) is found to be non-pathogenic to rabbits in the present investigation.

It may be that C. enzymicum was erroneously placed in the genus Corynebacterium by Eberson (1918). Carrier (1963) was the first to point out that C. enzymicum has little resemblance to the type species of the genus Corynebacterium and violates the generic diagnosis in that it is the only species which is catalase-negative and lacks the cytochrome oxidase. Unlike most corynebacteria it is very indifferent to oxygen, strongly fermentative and produces considerable acid from carbohydrates. He suggested that this species should be removed from the genus Corynebacterium and placed in the genus Lactobacillus.

A comparison of characteristics of C. enzymicum and selected species of the genera Corynebacterium, Lactobacillus and Microbacterium is presented in Table 23. The morphological characteristics of the strains (rod-shaped, gram-positive, non-motile, granules, etc.) are observed not only in the genus Corynebacterium, but also in Microbacterium and in Lactobacillus, and sometimes in other genera. The palisade arrangement frequently observed in C. diphtheriae and

Table 23. A comparison of characteristics of C. enzymicum and the genera Corynebacterium, Lactobacillus and Microbacterium.

Characteristic	C. enzymicum	C. diphtheriae	L. casei	M. lacticum
Oxygen relationship	Indifferent	Required	Not required	Required
Optimum growth temperature	30 - 33 C	37 C	30 C	32 C
Lethal temperature	60 C - 3 min	50 C - 3 min	?	Survives 85 C - 2½ min
Carbohydrate requirement	Essential	Not essential	Essential	Not essential
Glucose utilization	Fermentative	Oxidative	Fermentative	Oxidative and fermentative
Lactic acid production	+++	+	+++	+
Ribose utilization	Fermentative	Oxidative	Fermentative	Not utilized

Table 23. Continued.

Characteristic	*C. enzymicum*	*C. diphtheriae*	*L. casei*	*M. lacticum*
Litmus milk	Acid, coagulated, reduced	No change	Acid, coagulated, reduced	Slight acid
Nitrate reduction	±	+	-	±
Esculin hydrolysis	+	-	?	-
Catalase	-	+	-	+
Cytochrome oxidase	-	+	-	+

+, positive; -, negative; ?, not known.

M. lacticum is not found in C. enzymicum. The two strains of C. emzymicum form small coccoid rods which occur in small clumps or aggregates and in pairs but not in chains. L. casei is found mostly in short or long chains.

Physiologically and biochemically, however, several distinctive features are noticed among the organisms (Table 23). It is apparent that C. enzymicum differs significantly from the type species of the genus Corynebacterium. A close relationship between C. enzymicium and the Lactobacillus species, L. casei is apparent.

C. enzymicum seems to be totally indifferent to the effect of free oxygen. L. casei is anaerobic. Both of these organisms differ from the type species of the genera Corynebacterium and Microbacterium which are aerobic (Table 23). C. diphtheriae needs an abundance of oxygen for best growth; M. lacticum grows only slowly under anaerobic conditions. The optimum growth temperature of C. enzymicum is between 30 and 33 C, closely parallel to that of L. casei which is 30 C and that of M. lacticum which is 32 C. On the other hand, the optimum temperature of C. diphtheriae is 37 C.

C. enzymicum grows very poorly in ordinary media unless these are supplemented with fermentable sugars. The requirement of carbohydrates is also essential for the growth of the members of the genus Lactobacillus. C. diphtheriae and M. lacticum, on the other hand, do not need carbohydrates as essential supplements for growth.

C. enzymicum, like the lactobacilli, is strongly fermentative and ferments glucose, and similar aldehydic hexoses and carbohydrates which yield these simple sugars. Most of the members of the genus

Corynebacterium are highly oxidative and fermentation of carbohydrates by aerobic species of this genus is apparently limited (Frobisher, 1938; Welsch and Thibault, 1948). The type species, C. diphtheriae, utilizes glucose oxidatively (Carrier, 1963). The microbacteria utilize glucose oxidatively and fermentatively. Unlike the lactobacilli, they are aerobic and apparently obtain their energy by complete oxidation of fermentation products.

C. enzymicum, like the lactobacilli, seems to obtain its energy by the fermentation of carbohydrates to lactic acid as the sole end-product. The lactic acid produced by C. enzymicum appears to be entirely dextrorotatory (L+). Thus C. enzymicum resembles strongly the homofermentative lactobacilli. The microbacteria also produce dextrorotatory lactic acid, but the amount of lactic acid produced is very small and hence in this respect they differ significantly from the lactic acid bacteria (Orla-Jensen, 1919). According to Ramamurthi (1957), most species of the genus Corynebacterium produce only a small amount of acid from carbohydrates. A comparative study of the fermentative activity of some species of the genus Corynebacterium including the type species revealed that none of these produce a significant amount of lactic acid (Table 16 and Figures 14, 15).

Ribose is utilized fermentatively by both C. enzymicum and L. casei to produce lactic acid. C. diphtheriae also utilizes ribose but only oxidatively. The microbacteria, however, are not known to attack pentoses (Orla-Jensen, 1919).

Unlike most species of the genus Corynebacterium, which produce little change in milk (Carrier, 1963), C. enzymicum is found to

acidify and coagulate milk within 24 hours. Many of the lactobacilli also acidify and coagulate milk (Breed et al., 1957). The microbacteria generally produce insufficient acid to coagulate milk.

C. enzymicum apparently reduces a slight amount of nitrate to nitrite as is the case with M. lacticum. L. casei does not reduce nitrate. C. diphtheriae, however, easily reduces nitrate to nitrite.

C. enzymicum also differs from most of the animal species of the genus Corynebacterium (Carrier, 1963) in that it hydrolyzes esculin. The action of L. casei on esculin is not known.

All aerobic species of the genus Corynebacterium, except C. enzymicum form catalase and cytochrome oxidase. This singular feature may be considered as sufficient basis for removing C. enzymicum from the genus Corynebacterium. The organism in this respect presents a close relationship to the lactobacilli. By generic definition lactobacilli are all catalase-negative (Davis, 1964). The microbacteria, on the other hand, are all catalase-positive (Breed et al., 1957).

Leifson (1966), Schildkraut et al. (1962) and others have indicated the possible value of the DNA base composition of bacteria in taxonomy. According to Marmur et al. (1963), organisms which are closely related genetically or by the criteria of numerical taxonomy have DNA base compositions which are similar. The results of this study show that the DNA base composition of C. enzymicum is quite different from that of the other members of the genus Corynebacterium that have been reported in the literature (Table 24, from Marmur et al., 1963). The G-C content (mole % G + C) of the corynebacteria

Table 24. DNA base composition of some organisms similar to *C. enzymicum* (from Marmur *et al.*, 1963).

*Mole percent G + C	Organisms
38-40	*Diplococcus pneumoniae*, *Listeria monocytogenes*, *Streptococcus salivarius*, *S. pyogenes*, *S. bovis*, *S. cremoris*, *S. viridans*, *S. sanguis*, *Leuconostoc mesenteroides*, *Lactobacillus acidophilus*
46-48	*Corynebacterium acnes*, *C. pyogenes*
52-54	*Corynebacterium diphtheriae*, *C. cutis*, *C. avidum*, *C. granulosum*, *C. phocae*, *C. michiganese*, *C. facians*, *C. diphtheroides*
54-56	*Corynebacterium diphtheriae*
56-58	*Lactobacillus bifidus*, *Corynebacterium xerosis*, *C. vadosum*, *C. hofmanni*, *C. pseudodiphtheriticum*
58-60	*Corynebacterium parvum*, *C. ilicis*, *C. equi*

* $\frac{G + C}{A + T + G + C}$

are in the range of 46-60. Strains of the type species, C. diphtheriae have G-C content varying from 52-56. Only two species of the genus, C. acnes and C. pyogenes have G-C content of 46-48; most other species fall in the range of 52 to 54. On the other hand, the G-C content of C. enzymicum is found to be 39. Many of the lactic acid bacteria (streptococci, leuconostoc, lactobacillus, etc.) have G-C content of 38 to 40. Thus the DNA base composition suggests that C. enzymicum is more closely related to the genus Lactobacillus than to Corynebacterium. The DNA base composition of species of Microbacterium have not been reported.

From the morphological, physiological and biochemical studies along with studies on DNA base composition, it appears very reasonable to consider C. enzymicum as a lactic acid bacterium, closely related to the Lactobacillus group. Comparative study of C. enzymicum with the type species of the genera Corynebacterium and Microbacterium and a representative member of the genus Lactobacillus indicates that C. enzymicum is more closely related to the species of the genus Lactobacillus than to the other organisms. The author agrees with the suggestions of Carrier (1963) that C. enzymicum should be removed from the genus Corynebacterium and placed in the genus Lactobacillus. It differs, however, from other described species of that genus, hence it should be called Lactobacillus enzymicus.

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EXAMINATION AND THESIS REPORT

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Major Field: Microbiology

Title of Thesis: Studies on Corynebacterium enzymicum (Mellon) Eberson

Approved:

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